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<b>(21) International Application Number:</b> PCT/US98/22655 <b>(22) International Filing Date:</b> 26 October 1998 (26.10.98)  <b>(30) Priority Data:</b> 60/063,624                      27 October 1997 (27.10.97)                      US  <b>(71) Applicant (for all designated States except US):</b> CREATIVE BIOMOLECULES, INC. [US/US]; 45 South Street, Hopkinton, MA 01748 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CHARETTE, Marc, F. [US/US]; 18 Ellicott Street, Needham, MA 02192 (US). RUEGER, David, C. [US/US]; 81 Pine Hill Road, Southborough, MA 01772 (US). HIGGINS, Dennis [US/US]; 56 Hilton Boulevard, Amherst, NY 14226 (US).  <b>(74) Agent:</b> LINKKILA, Timothy, P.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> ENHANCEMENT OF MORPHOGEN ACTIVITY  <b>(57) Abstract</b>  <p>The present invention provides methods and compositions for enhancing morphogen activity in mammals, including humans. The methods involve the administration of a molecule capable of releasing morphogen inhibition. The molecule can be administered either alone or in combination with a morphogen. The present invention further provides screening methods for indentifying a molecule capable of enhancing morphogen activity.</p>		

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## ENHANCEMENT OF MORPHOGEN ACTIVITY

### Field of the Invention

The present invention relates to enhancement of morphogen activity. The methods involve the administration of a molecule capable of releasing morphogen inhibition. The present invention further provides screening methods for identifying a molecule capable of enhancing morphogen activity.

### Background of the Invention

Morphogens have been shown to induce tissue-specific morphogenesis in mammals. These proteins are able, on their own, to induce the migration, proliferation and differentiation of progenitor cells into functional replacement tissue. Although morphogens were initially recognized for their ability to induce ectopic, endochondral bone morphogenesis, these proteins have been shown to have utility in repairing a number of non-chondrogenic tissues, including dentin, liver, kidney, neural, cardiac lung, epithelial, and gastrointestinal tissue. *See*, for example, WO 92/15323; WO 93/04692; WO 94/06399; WO 94/03200; WO 94/06449; and WO 94/06420. *See also*, USSN 08/404,113; 08/445,467; 08/432,883; 08/155,343; 08/260675; 08/445,468; 08/461,397; 08/480,528; 08/402,542; 08/396,930; and 08/751,227, the disclosures of which are incorporated by reference.

The morphogenic activities of these proteins allow them to initiate and maintain the developmental cascade of tissue morphogenesis in an appropriate, morphogenically-permissive environment, stimulating stem cells to proliferate and differentiate in a tissue-specific manner, and inducing the progression of events that culminate in new tissue formation. These morphogenic activities also allow the proteins to stimulate the "redifferentiation" of cells previously induced to stray from their differentiation path. The proteins are useful in the replacement of diseased or damaged tissue in a mammal, particularly when the damaged tissue interferes with normal tissue or organ function, such as, for example, damaged lung tissue resulting from emphysema; cirrhotic kidney or liver tissues; damaged heart or blood vessel tissue, as may result from cardiomyopathies and/or atherothrombotic or cardioembolic strokes; damaged stomach tissue resulting from ulceric perforations or their repair; damaged neural tissue as may result from physical or chemical injury,

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such as strokes, or neuropathies such as Alzheimer's disease, Parkinson's disease, Huntington's chorea, or multiple sclerosis; damaged skeletal or orthopedic tissues, dentin and periodontal tissues as may result from disease or mechanical injury.

As described herein, various factors have been shown to inhibit morphogen activity in mammalian cells. The inhibitory effects of these endogenously-released factors were observed in the presence of optimal concentrations of the morphogen. As such, these factors may compromise the ability of endogenous or exogenous morphogen to replace diseased or damaged tissues. Accordingly, there is a need in the art for methods and compositions capable of releasing this morphogen inhibition.

### Summary of the Invention

The present invention provides methods for potentiating endogenous morphogen activity, including methods for promoting neuronal cell growth and methods for treating a disorder characterized by neuronal cell loss. These methods comprise administering a composition comprising a molecule capable of releasing inhibition on morphogen activity. Morphogen inhibition may be in the form of endogenous inhibitory compounds, such as leukemia inhibitory factor or cytokines, or may be in the form of exogenously applied inhibitors. The present invention further provides methods for treating a neurodegenerative disorder comprising administering a composition comprising a morphogen and a molecule capable of releasing morphogen inhibition. The methods and compositions of the invention may be used to treat various injuries to and/or deficiencies in neural pathways and neuronal cells, including those injuries that result from trauma or diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, senile dementia, alcohol-induced dementia, and stroke. In particular, the methods are useful for releasing inhibition of morphogen activity in order to maintain neural cell phenotype and functional neural pathways. The methods are particularly useful where treatment demands neurite outgrowth (and especially dendritic outgrowth) to achieve proper neural phenotype or neural pathway function.

In a preferred embodiment, a molecule capable of releasing morphogen inhibition is a cytokine antagonist, a leukemia inhibitory factor antagonist, a ciliary neurotrophic factor or a retinoid antagonist. In a particularly preferred embodiment, the cytokine antagonist is an antagonist of a neuropoietic cytokine. In other particularly preferred embodiments, the retinoid

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antagonist administered in the composition is a retinoic acid receptor antagonist, or a retinoid X receptor antagonist.

In methods comprising the administration of a morphogen, a preferred morphogen comprises an amino acid sequence selected from the group consisting of a sequence having 70%  
5 homology with the C-terminal seven-cysteine skeleton of human OP-1 (amino acids 330-341 of SEQ ID NO: 2); a sequence having greater than 60% amino acid sequence identity with human OP-1; generic sequence 7 (SEQ ID NO: 4); generic sequence 8 (SEQ ID NO: 5); generic sequence 9 (SEQ ID NO: 6); generic sequence 10 (SEQ ID NO: 7); and OPX (SEQ ID NO: 3). Specifically, the morphogen is selected from the group consisting of human OP-1, mouse OP-1,  
10 human OP-2, mouse OP-2, 60A, GDF-1, BMP2A, BMP2B, DPP, Vgl, Vgr-1, BMP3, BMP5, and BMP6.

In another aspect, the invention features methods for potentiating morphogen activity comprising the step of administering to a mammal a composition comprising a molecule that binds an endogenous ligand for a receptor selected from the group consisting of a cytokine receptor and  
15 a retinoid receptor. In a preferred embodiment, the cytokine receptor is a neuropoetic cytokine receptor. In a particularly preferred embodiment, the neuropoetic cytokine receptor is either an LIF receptor or a CTNF receptor. In an alternative preferred embodiment, the retinoid receptor is a retinoic acid receptor.

In another aspect, the invention features methods for potentiating morphogen activity by  
20 administering to a mammal a composition comprising a cyclic AMP (cAMP) dependent messenger pathway inhibitor. In a preferred embodiment, the cAMP-dependent messenger pathway inhibitor is a protein kinase A inhibitor. Such inhibitors include (2-p-bromocynnamylaminoethyl)-5-isoquinolinesulfonamide and sterically constrained enantiomers of dibutyryl cAMP and cAMP.

25 In another aspect, the invention features screening methods for identifying a molecule capable of potentiating morphogen activity or releasing inhibition of morphogen activity. The methods comprise: (1) exposing a cell to a candidate molecule; (2) determining whether the cell exhibits increased morphogenic activity; and (3) identifying a molecule capable of potentiating morphogenic activity as a candidate that induces an increase in morphogen activity in the cell.

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In yet another aspect, the invention provides molecules identified by screening methods of the present invention. These molecules include proteins, other organic molecules, and inorganic molecules.

As used herein, the terms "morphogen," "bone morphogen," "bone morphogenic protein," "BMP," "morphogenic protein" and "morphogenetic protein" all embrace the class of proteins typified by human osteogenic protein 1 (hOP-1). Nucleotide and amino acid sequences for hOP-1 are provided in SEQ ID NOS: 1 and 2, respectively. For ease of description, hOP-1 is considered a representative morphogen. It will be appreciated that OP-1 is merely representative of the TGF- $\beta$  subclass of true tissue morphogens and is not intended to limit the description. Other known and useful morphogens include, but are not limited to, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-15, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, 60A, NODAL, UNIVIN, SCREW, ADMP, and NEURAL, and morphogenically-active amino acid variants of any thereof.

In specific embodiments, useful morphogens include those sharing the conserved seven cysteine skeleton, and sharing at least 70% amino acid sequence homology (similarity), within the C-terminal seven-cysteine skeleton of human OP-1, residues 330-431 of SEQ ID NO: 2 (hereinafter referred to as the presently-preferred reference sequence). In another embodiment, the invention encompasses use of biologically active species (phylogenetic) variants of any of the morphogenic proteins recited herein, including conservative amino acid sequence variants, proteins encoded by degenerate nucleotide sequence variants, and morphogenically-active proteins sharing the conserved seven cysteine skeleton as defined herein and encoded by a DNA sequence competent to hybridize under standard stringency conditions to a DNA sequence encoding a morphogenic protein disclosed herein, including, without limitation, OP-1 or BMP-2 or BMP-4. Presently, however, the preferred reference sequence is that of residues 330-431 of SEQ ID NO: 2 (OP-1).

In still another embodiment, morphogens useful in methods and compositions of the invention are defined as morphogenically-active proteins having any one of the generic sequences defined herein, including OPX (SEQ ID NO: 3) and Generic Sequences 7 and 8 (SEQ ID NOS: 4 and 5, respectively), or Generic Sequences 9 and 10 (SEQ ID NOS: 6 and 7, respectively). OPX

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encompasses the observed variation between the known phylogenetic counterparts of the osteogenic OP-1 and OP-2 proteins, and is described by the amino acid sequence presented herein below and in SEQ ID NO: 3. Generic Sequence 9 is a 96 amino acid sequence containing the C-terminal six cysteine skeleton observed in hOP-1 (residues 335-431 of SEQ ID NO: 2) and  
5 wherein the remaining residues encompass the observed variation among OP-1, OP-2, OP-3, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-8, BMP-9, BMP-10, BMP-11, BMP-15, GDF-1, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, 60A, UNIVIN, NODAL, DORSALIN, NEURAL, SCREW and ADMP. That is, each of the non-cysteine residues is independently selected from the corresponding residue in this recited group of known,  
10 naturally-sourced proteins. Generic Sequence 10 is a 102 amino acid sequence which includes a five amino acid sequence added to the N-terminus of the Generic Sequence 9 and defines the seven cysteine skeleton observed in hOP-1 (330-431 SEQ ID NO: 2). Generic Sequences 7 and 8 are 96 and 102 amino acid sequences, respectively, containing either the six cysteine skeleton (Generic Sequence 7) or the seven cysteine skeleton (Generic Sequence 8) defined by hOP-1 and  
15 wherein the remaining non-cysteine residues encompass the observed variation among OP-1, OP-2, OP-3, BMP-2, BMP-3, BMP-4, 60A, DPP, Vg1, BMP-5, BMP-6, Vgr-1, and GDF-1.

Of particular interest herein are morphogens which, when provided to a specific tissue of a mammal, induce tissue-specific morphogenesis or maintain the normal state of differentiation and growth of that specific tissue. In preferred demonstrative embodiments, the present morphogens  
20 induce the formation of vertebrate (*e.g.*, avian or mammalian) body tissues, such as but not limited to nerve, eye, bone, cartilage, bone marrow, ligament, tooth dentin, periodontium, liver, kidney, lung, heart, or gastrointestinal lining. The present demonstrations can be carried out in the context of developing embryonic tissue, or at an aseptic, unscarred wound site in post-embryonic tissue. Methods of identifying such morphogens, or morphogen receptor  
25 agonists, are known in the art and include assays for compounds which induce morphogen-mediated responses (*e.g.*, induction of endochondral bone formation, induction of differentiation of metanephric mesenchyme, and the like). In a currently preferred demonstrative embodiment, morphogens of the present invention, when implanted in a mammal in conjunction with a matrix permissive of bone morphogenesis, are capable of inducing a developmental cascade  
30 of cellular and molecular events that culminates in endochondral bone formation. See U.S.

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4,968,590 and Sampath, *et al.*, *Proc. Natl. Acad. Sci. USA* 80: 6591-6595 (1983), the disclosures of which are incorporated herein by reference.

### Brief Description of the Drawings

FIG. 1 is a tabular presentation of the percent amino acid sequence identity and percent amino acid sequence homology ("similarity") that various members of the family of morphogenic proteins as defined herein share with hOP-1 in the C-terminal seven cysteine skeleton;

FIG. 2 (Panels A-C) are line graphs depicting time course of the response of cultured sympathetic neurons to LIF and OP-1. Beginning on the fifth day, cells were treated with OP-1 (50 ng/ml), LIF (30 ng/ml), or with a combination of OP-1 (50 ng/ml) and LIF (30 ng/ml) during the time course study. Intracellular dye injections were performed at various times to determine: (i) the percentage of cells with dendrites (Panel A); (ii) the mean number of dendrites/cell (Panel B); (iii) the length of the longest dendrite (Panel C); and (iv) the number of cells/culture and the number of axons/cell (Panel D). Open circles, control; filled circles, OP-1; open squares, LIF; filled squares, OP-1 + LIF. Data are expressed as mean  $\pm$  SEM.  $N \geq 30$ .

FIG. 3 is a line graph depicting the time course of the response of the inhibitory effect of LIF on OP-1-induced dendritic growth in cultured sympathetic neurons. LIF (30 ng/ml) was added at 3, 5, or 7 days to cultured sympathetic neurons treated with OP-1 (50 ng/ml). Filled circles, OP-1; open circles OP-1 + LIF. Data are expressed as mean  $\pm$  SEM.  $N \geq 30$ .

FIG. 4 is a line graph depicting the effects of varying concentrations of LIF and CNTF on dendritic growth and OP-1-stimulated dendritic growth. To assess the inhibitory effects on dendritic growth, sympathetic neurons were exposed to OP-1 (50 ng/ml) and varying concentrations of LIF (open circles) and CNTF (filled circles) for five days. The stimulatory effects of LIF (open squares) and CNTF (filled squares) on dendritic growth have a longer latency and were assessed on day 10. Data are expressed as mean  $\pm$  SEM.  $N \geq 30$ .

FIG. 5 is a bar graph depicting anti-LIF antibody blocking the inhibitory effects of LIF on OP-1-stimulated dendritic growth. Exposure to OP-1 (50 ng/ml) from the fifth to the tenth day *in vitro* induced dendritic growth which was unaffected by the presence of antibody to LIF. OP-1-induced dendritic growth was reduced by 58.8% in the presence of LIF (1 ng/ml) and this inhibition was unaffected by the addition of non-immune IgG (30  $\mu$ g/ml). However, 10 and



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30 µg/ml anti-LIF antibody reversed the LIF-induced inhibition of OP-1-stimulated dendritic growth in a dose-dependent manner. Data are expressed as mean  $\pm$  SEM.  $N \geq 30$ . \* $p < 0.01$  vs no antibody.

FIG. 6 is a line graph depicting the effects of varying concentrations of retinoic acid on dendritic growth and OP-1-stimulated dendritic growth. To assess the inhibitory effects on dendritic growth, sympathetic neurons were exposed to varying concentrations of retinoic acid (open squares) or to OP-1 (50 ng/ml) and varying concentrations of retinoic acid (open circles) for five days. Data are expressed as mean  $\pm$  SEM.  $N \geq 30$ .

FIG. 7 is a line graph depicting the effects of various concentrations of retinoic acid on dendritic growth and OP-1 stimulated dendritic growth.

FIG 8A is a line graph depicting the effects of OP-1 alone (dark square, 50 ng/ml) or OP-1 plus various concentrations of forskolin (dark circles) or db-cAMP (open circles) on the number of dendrites present in cultures of rat sympathetic neurons. Mean  $\pm$  S.E.M. ( $N=30$ ).

FIG. 8B is a line graph depicting the effects of OP-1 alone (dark square, 50ng/ml) or OP-1 plus various concentrations of forskolin (dark circles) or db-cAMP (open circles) on the length of the longest dendrite (µm) in cultures of rat sympathetic neurons. Mean  $\pm$  S.E.M. ( $N=30$ ).

FIG. 9 is a bar graph depicting the effects on cultures of sympathetic neurons exposed to OP-1 (50 ng/ml) for 3 days, then subsequently exposed to OP-1 plus CNTF (30 ng/ml) or LIF (30 ng/ml). Other cultures were treated with PI-PLC (1 U/ml) for 1 hour before treatment with CNTF or LIF. Mean  $\pm$  S.E.M. ( $N=30$ ). \* $P > 0.01$  vs OP-1=CNTF.

### Detailed Description of Preferred Embodiments

#### A. General

Various endogenous factors are known to inhibit morphogen activity in mammalian cells. The inhibitory effects of these endogenously-released factors are observed in the presence of optimal concentrations of the morphogen. As such, these factors can compromise the ability of endogenous or exogenous morphogen to replace diseased or damaged tissues. Accordingly, the present invention provides methods for identifying molecules capable of releasing this morphogen inhibition. In the context of the present application, "releasing" means reducing or suppressing.

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It is expected that the invention will be useful for enhancing morphogen activity. It is also expected that the invention will be useful for preserving or restoring function of a tissue damaged by mechanical or chemical trauma, or a disease.

**B. Biochemical, Structural and Functional Properties of Useful Morphogenic Proteins**

As noted above, molecules capable of releasing this morphogen inhibition can be administered alone or with a morphogen. As defined herein, a protein is morphogenic if it induces the developmental cascade of cellular and molecular events that culminate in the formation of new, organ-specific tissue. In one preferred embodiment, a morphogen is a dimeric protein, each polypeptide component of which has a sequence that corresponds to, or is functionally equivalent to, at least the conserved C-terminal six or seven cysteine skeleton of human OP-1, included in SEQ ID NO: 5, and/or which shares 70% amino acid sequence homology with OP-1 in this region. The morphogens are generally competent to induce a cascade of events including all of the following, in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated cells; and supporting the growth and maintenance of differentiated cells. Under appropriate conditions the morphogens are also competent to induce redifferentiation of committed cells, particularly of cells that have strayed from their "normal" differentiation pathway. Details of how the morphogens useful in this invention were identified, as well as a description on how to make, use and test them for morphogenic activity are disclosed in numerous publications, including U.S. Patent Nos. 5,011,691 and 5,266,683, and the international patent application publications WO 92/15323; WO 93/04692; and WO 94/03200 each of which are incorporated by reference herein. As disclosed therein, the morphogens can be purified from naturally-sourced material or recombinantly produced from prokaryotic or eukaryotic host cells, using the genetic sequences disclosed therein. Alternatively, novel morphogenic sequences can be identified following the procedures disclosed therein.

The naturally-occurring morphogens share substantial amino acid sequence homology in their C-terminal sequences (sharing *e.g.*, a six or seven cysteine skeleton sequence). Typically, a naturally-occurring morphogen is translated as a precursor, having an N-terminal signal peptide sequence, typically less than about 35 residues in length, followed by a "pro" domain that is

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cleaved to yield the mature polypeptide, which includes the biologically active C-terminal skeleton sequence. The signal peptide is cleaved rapidly upon translation, at a cleavage site that can be predicted in a given sequence using the method of Von Heijne, *Nucleic Acids Research* 14: 4683-4691 (1986). The pro polypeptide typically is about three times larger than the fully processed, mature C-terminal polypeptide. Under native conditions, the protein is secreted as a mature dimer and the cleaved pro polypeptide is thought to remain associated therewith to form a protein complex, presumably to improve the solubility of the mature dimeric protein. The complexed form of a morphogen is generally observed to be more soluble than the mature form under physiological conditions.

10 Natural-sourced morphogenic protein in its mature, native form, typically is a glycosylated dimer, typically having an apparent molecular weight of about 30-36 kDa as determined by SDS-PAGE. When reduced, the 30 kDa protein gives rise to two glycosylated polypeptide subunits having apparent molecular weights in the range of about 16 kDa and about 18 kDa. The unglycosylated dimeric protein, which also has morphogenic activity, typically has an apparent  
15 molecular weight in the range of about 27 kDa. When reduced, the 27 kDa protein gives rise to two unglycosylated polypeptides having molecular weights typically in the range of about 14 kDa to about 16 kDa.

In preferred embodiments, each of the polypeptide chains of a dimeric morphogenic protein as defined herein comprises an amino acid sequence sharing a defined relationship with an amino acid sequence of a reference morphogen. In one embodiment, preferred morphogenic polypeptide chains share a defined relationship with a sequence present in morphogenically-active human OP-1, SEQ ID NO: 2. However, any one or more of the naturally-occurring or biosynthetic morphogenic proteins disclosed herein similarly could be used as a reference sequence. Preferred morphogenic polypeptide chains share a defined relationship with at least the  
20 C-terminal six cysteine skeleton of human OP-1, residues 335-431 of SEQ ID NO: 2. Preferably, morphogenic polypeptide chains share a defined relationship with at least the C-terminal seven cysteine skeleton of human OP-1, residues 330-431 of SEQ ID NO: 2. That is, preferred polypeptide chains in a dimeric protein with tissue morphogenic activity each comprise a sequence that corresponds to a reference sequence or is functionally equivalent thereto.

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Functionally equivalent sequences include functionally equivalent arrangements of cysteine residues disposed within the reference sequence, including amino acid insertions or deletions which alter the linear arrangement of these cysteines, but do not materially impair their relationship in the folded structure of the dimeric morphogen protein, including their ability to form such intra- or inter-chain disulfide bonds as may be necessary for morphogenic activity. For example naturally-occurring morphogens have been described in which at least one internal deletion (of one residue; BMP2) or insertion (of four residues; GDF-1) is present but does not abrogate biological activity. Functionally equivalent sequences further include those wherein one or more amino acid residues differ from the corresponding residue of a reference sequence, *e.g.*, the C-terminal seven cysteine skeleton of human OP-1, provided that this difference does not destroy tissue morphogenic activity. Accordingly, conservative substitutions of corresponding amino acids in the reference sequence are preferred. Amino acid residues that are "conservative substitutions" for corresponding residues in a reference sequence are those that are physically or functionally similar to the corresponding reference residues, *e.g.*, that have similar size, shape, electric charge, chemical properties including the ability to form covalent or hydrogen bonds, or the like. Particularly preferred conservative substitutions are those fulfilling the criteria defined for an accepted point mutation in Dayhoff, *et al.*, 5 ATLAS OF PROTEIN SEQUENCE AND STRUCTURE, Suppl. 3, ch. 22 pp. 354-352 (1978), Natl. Biomed. Res. Found., Washington, D.C. 20007, the teachings of which are incorporated by reference herein. Examples of conservative substitutions include the substitution of one amino acid for another with similar characteristics, *e.g.*, substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The term "conservative substitution" also includes the use of a synthetic or derivatized amino acid in place of the corresponding natural parent amino acid, provided that antibodies raised to the resulting variant polypeptide also immunoreact with the corresponding naturally sourced morphogen polypeptide.

The following publications disclose publications morphogen polypeptide sequences, as well as relevant chemical and physical properties, of naturally-occurring and/or synthetic morphogens: OP-1 and OP-2: U.S. 5,011,691, U.S. 5,266,683, Ozkaynak, *et al.*, *EMBO J.* 9: 2085-2093 (1990); OP-3: WO 94/10203 (PCT US93/10520); BMP-2, BMP-3, and BMP-4: WO 88/00205, Wozney, *et al.*, *Science* 242: 1528-1534 (1988); BMP-5 and BMP-6: Celeste, *et*

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*al.*, *PNAS* 87: 9843-9847 (1991); Vgr-1: Lyons, *et al.*, *PNAS* 86: 4554-4558 (1989); DPP: Padgett, *et al.*, *Nature* 325: 81-84 (1987); Vg-1: Weeks *Cell* 51: 861-867 (1987); BMP-9: WO 95/33830 (PCT/US95/07084); BMP-10: WO 94/26893 (PCT/US94/05290); BMP-11: WO 94/26892 (PCT/US94/05288); BMP-12: WO 95/16035 (PCT/US94/14030); BMP-13: 5 WO 95/16035 (PCT/US94/14030); GDF-1: WO 92/00382 (PCT/US91/04096) and Lee, *et al.*, *PNAS* 88: 4250-4254 (1991); GDF-8: WO 94/21681 (PCT/US94/03019); GDF-9: WO 94/15966 (PCT/US94/00685); GDF-10: WO 95/10539 (PCT/US94/11440); GDF-11: WO 96/01845 (PCT/US95/08543); BMP-15: WO 96/36710 (PCT/US96/06540); *MP121*: WO 96/01316 (PCT/EP95/02552); GDF-5 (CDMP-1, MP52): WO 94/15949 (PCT/US94/00657) and 10 WO 96/14335 (PCT/US94/12814) and WO 93/16099 (PCT/EP93/00350); GDF-6 (CDMP-2, BMP-13): WO 95/01801 (PCT/US94/07762) and WO 96/14335 and WO 95/10635 (PCT/US94/14030); GDF-7 (CDMP-3, BMP-12): WO 95/10802 (PCT/US94/07799) and WO 95/10635 (PCT/US94/14030). In another embodiment, useful proteins include biologically active biosynthetic constructs, including novel biosynthetic morphogenic proteins and chimeric 15 proteins designed using sequences from two or more known morphogens. See also the biosynthetic constructs disclosed in U.S. Pat. 5,011,691, the disclosure of which is incorporated herein by reference (*e.g.*, COP-1, COP-3, COP-4, COP-5, COP-7, and COP-16).

In certain preferred embodiments, useful morphogenic proteins include those in which the amino acid sequences comprise a sequence sharing at least 70% amino acid sequence homology 20 or "similarity", and preferably 80% homology or similarity, with a reference morphogenic protein selected from the exemplary, naturally-occurring morphogenic proteins listed herein. Preferably, the reference protein is human OP-1, and the reference sequence thereof is the C-terminal seven cysteine skeleton present in osteogenically active forms of human OP-1, residues 330-431 of SEQ ID NO: 2. Useful morphogenic proteins accordingly include allelic, phylogenetic counterpart and 25 other variants of the preferred reference sequence, whether naturally-occurring or biosynthetically produced (*e.g.*, including "muteins" or "mutant proteins"), as well as novel members of the general morphogenic family of proteins including those set forth and identified above. Certain particularly preferred morphogenic polypeptides share at least 60% amino acid identity with the preferred reference sequence of human OP-1, still more preferably at least 65% amino acid 30 identity therewith.

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In certain embodiments, a polypeptide suspected of being functionally equivalent to a reference morphogen polypeptide is aligned therewith using the method of Needleman, *et al.*, *J. Mol. Biol.* 48: 443-453 (1970), implemented conveniently by computer programs such as the Align program (DNASTar, Inc.). As noted above, internal gaps and amino acid insertions in the candidate sequence are ignored for purposes of calculating the defined relationship, conventionally expressed as a level of amino acid sequence homology, or identity, between the candidate and reference sequences. "Amino acid sequence homology" is understood herein to include both amino acid sequence identity and similarity. Homologous sequences share identical and/or similar amino acid residues, where similar residues are conservation substitutions for, or "allowed point mutations" of, corresponding amino acid residues in an aligned reference sequence. Thus, a candidate polypeptide sequence that shares 70% amino acid homology with a reference sequence is one in which any 70% of the aligned residues are either identical to, or are conservative substitutions of, the corresponding residues in a reference sequence. In a currently preferred embodiment, the reference sequence is the C-terminal seven cysteine skeleton sequence of human OP-1.

FIG. 1 recites the percent amino acid sequence homology (similarity) and percent identity within the C-terminal seven cysteine skeletons of various representative members of the TGF- $\beta$  family, using OP-1 as the reference sequence. The percent homologies recited in the figure are determined by aligning the sequences essentially following the method of Needleman, *et al.*, *J. Mol. Biol.*, 48: 443-453 (1970), and using the Align Program (DNASTar, Inc.). Insertions and deletions from the reference morphogen sequence (the C-terminal, biologically active seven-cysteine skeleton of hOP-1) are ignored for purposes of calculation.

As is apparent to one of ordinary skill in the art reviewing the sequences for the proteins listed in FIG. 1, significant amino acid changes can be made from the reference sequence while retaining substantial morphogenic activity. For example, while the GDF-1 protein sequence shares only about 50% amino acid identity with the hOP-1 sequence described herein, the GDF-1 sequence shares greater than 70% amino acid sequence homology with the hOP-1 sequence, where "homology" is as defined above. Moreover, GDF-1 contains a four amino acid insert (Gly-Gly-Pro-Pro) between the two residues corresponding to residue 372 and 373 of OP-1 (SEQ ID NO: 2). Similarly, BMP-3 has a "extra" residue, a valine, inserted between the two residues

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corresponding to residues 385 and 386 of hOP-1 (SEQ ID NO: 2). Also, BMP-2 and BMP-4 are both "missing" the amino acid residue corresponding to residue 389 of OP-1 (SEQ ID NO: 2). None of these "deviations" from the reference sequence appear to interfere substantially with biological activity.

5           In other preferred embodiments, the family of morphogenic polypeptides useful in the present invention, and members thereof, are defined by a generic amino acid sequence. For example, Generic Sequence 7 (SEQ ID NO: 4) and Generic Sequence 8 (SEQ ID NO: 5) disclosed below, encompass the observed variations between preferred protein family members identified to date, including at least OP-1, OP-2, OP-3, CBMP-2A, CBMP-2B, BMP-3, 60A,  
10   DPP, Vg1, BMP-5, BMP-6, Vgr-1, and GDF-1. The amino acid sequences for these proteins are described herein and/or in the art, as summarized above. The generic sequences include both the amino acid identity shared by these sequences in the C-terminal skeleton, defined by the six and seven cysteine skeletons (Generic Sequences 7 and 8, respectively), as well as alternative residues for the variable positions within the sequence. The generic sequences provide an appropriate  
15   cysteine skeleton where inter- or intramolecular disulfide bonds can form, and contain certain critical amino acids likely to influence the tertiary structure of the folded proteins. In addition, the generic sequences allow for an additional cysteine at position 36 (Generic Sequence 7) or position 41 (Generic Sequence 8), thereby encompassing the morphogenically-active sequences of OP-2 and OP-3.

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**Generic Sequence 7 (SEQ ID NO: 4)**

			Leu 1	Xaa	Xaa	Xaa	Phe 5	Xaa	Xaa
Xaa	Gly	Trp 10	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Pro
Xaa	Xaa	Xaa 20	Xaa	Ala	Xaa	Tyr	Cys 25	Xaa	Gly
Xaa	Cys	Xaa 30	Xaa	Pro	Xaa	Xaa	Xaa 35	Xaa	Xaa
Xaa	Xaa	Xaa 40	Asn	His	Ala	Xaa	Xaa 45	Xaa	Xaa
Xaa	Xaa	Xaa 50	Xaa	Xaa	Xaa	Xaa	Xaa 55	Xaa	Xaa
Xaa	Xaa	Xaa 60	Cys	Cys	Xaa	Pro	Xaa 65	Xaa	Xaa
Xaa	Xaa	Xaa 70	Xaa	Xaa	Leu	Xaa	Xaa 75	Xaa	Xaa
Xaa	Xaa	Xaa 80	Val	Xaa	Leu	Xaa	Xaa 85	Xaa	Xaa
Xaa	Met	Xaa 90	Val	Xaa	Xaa	Cys	Xaa 95	Cys	Xaa

wherein each Xaa independently is selected from a group of one or more specified amino acids defined as follows: "Res. " means "residue" and Xaa at res. 2 = (Tyr or Lys); Xaa at res. 3 = Val or Ile); Xaa at res. 4 = (Ser, Asp or Glu); Xaa at res. 6 = (Arg, Gln, Ser, Lys or Ala); Xaa at res. 7 = (Asp or Glu); Xaa at res. 8 = (Leu, Val or Ile); Xaa at res. 11 = (Gln, Leu, Asp, His, Asn or Ser); Xaa at res. 12 = (Asp, Arg, Asn or Glu); Xaa at res. 13 = (Trp or Ser); Xaa at res. 14 = (Ile or Val); Xaa at res. 15 = (Ile or Val); Xaa at res. 16 = (Ala or Ser); Xaa at res. 18 = (Glu, Gln, Leu, Lys, Pro or Arg); Xaa at res. 19 = (Gly or Ser); Xaa at res. 20 = (Tyr or Phe); Xaa at res. 21 = (Ala, Ser, Asp, Met, His, Gln, Leu or Gly); Xaa at res. 23 = (Tyr, Asn or Phe); Xaa at res. 26 = (Glu, His, Tyr, Asp, Gln, Ala or Ser); Xaa at res. 28 = (Glu, Lys, Asp, Gln or Ala); Xaa at res. 30 = (Ala, Ser, Pro, Gln, Ile or Asn); Xaa at res. 31 = (Phe, Leu or Tyr); Xaa at res. 33 = (Leu, Val or Met); Xaa at res. 34 = (Asn, Asp, Ala, Thr or Pro); Xaa at res. 35 = (Ser, Asp, Glu, Leu, Ala or Lys); Xaa at res. 36 = (Tyr, Cys, His, Ser or Ile); Xaa at res. 37 = (Met, Phe, Gly or Leu); Xaa at res. 38 = (Asn, Ser or Lys); Xaa at res. 39 = (Ala, Ser, Gly or Pro); Xaa at res. 40 = (Thr, Leu or Ser); Xaa at res. 44 = (Ile, Val or Thr); Xaa at res. 45 = (Val, Leu, Met or Ile); Xaa at res. 46 = (Gln or Arg); Xaa at res. 47 = (Thr, Ala or Ser); Xaa at res. 48 = (Leu or Ile); Xaa at res. 49 = (Val or Met); Xaa at res. 50 = (His, Asn or Arg); Xaa at res. 51 = (Phe, Leu, Asn, Ser, Ala or Val); Xaa at res. 52 = (Ile, Met, Asn, Ala, Val, Gly or Leu); Xaa at res. 53 = (Asn, Lys,



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Ala, Glu, Gly or Phe); Xaa at res. 54 = (Pro, Ser or Val); Xaa at res. 55 = (Glu, Asp, Asn, Gly, Val, Pro or Lys); Xaa at res. 56 = (Thr, Ala, Val, Lys, Asp, Tyr, Ser, Gly, Ile or His); Xaa at res. 57 = (Val, Ala or Ile); Xaa at res. 58 = (Pro or Asp); Xaa at res. 59 = (Lys, Leu or Glu); Xaa at res. 60 = (Pro, Val or Ala); Xaa at res. 63 = (Ala or Val); Xaa at res. 65 = (Thr, Ala or Glu);

5 Xaa at res. 66 = (Gln, Lys, Arg or Glu); Xaa at res. 67 = (Leu, Met or Val); Xaa at res. 68 = (Asn, Ser, Asp or Gly); Xaa at res. 69 = (Ala, Pro or Ser); Xaa at res. 70 = (Ile, Thr, Val or Leu); Xaa at res. 71 = (Ser, Ala or Pro); Xaa at res. 72 = (Val, Leu, Met or Ile); Xaa at res. 74 = (Tyr or Phe); Xaa at res. 75 = (Phe, Tyr, Leu or His); Xaa at res. 76 = (Asp, Asn or Leu); Xaa at res. 77 = (Asp, Glu, Asn, Arg or Ser); Xaa at res. 78 = (Ser, Gln, Asn, Tyr or Asp); Xaa at

10 res. 79 = (Ser, Asn, Asp, Glu or Lys); Xaa at res. 80 = (Asn, Thr or Lys); Xaa at res. 82 = (Ile, Val or Asn); Xaa at res. 84 = (Lys or Arg); Xaa at res. 85 = (Lys, Asn, Gln, His, Arg or Val); Xaa at res. 86 = (Tyr, Glu or His); Xaa at res. 87 = (Arg, Gln, Glu or Pro); Xaa at res. 88 = (Asn, Glu, Trp or Asp); Xaa at res. 90 = (Val, Thr, Ala or Ile); Xaa at res. 92 = (Arg, Lys, Val, Asp, Gln or Glu); Xaa at res. 93 = (Ala, Gly, Glu or Ser); Xaa at res. 95 = (Gly or Ala) and Xaa at res. 97 =

15 (His or Arg).

Generic Sequence 8 (SEQ ID NO: 5) includes all of Generic Sequence 7 (SEQ ID NO: 4) and in addition includes the following sequence (SEQ ID NO: 8) at its N-terminus:

#### SEQ ID NO: 8

Cys	Xaa	Xaa	Xaa	Xaa
1				5

Accordingly, beginning with residue 7, each "Xaa" in Generic Sequence 8 is a specified amino acid defined as for Generic Sequence 7, with the distinction that each residue number described for Generic Sequence 7 is shifted by five in Generic Sequence 8. Thus, "Xaa at res. 2 =(Tyr or Lys)" in Generic Sequence 7 refers to Xaa at res. 7 in Generic Sequence 8. In Generic Sequence 8, Xaa at res. 2 = (Lys, Arg, Ala or Gln); Xaa at res. 3 = (Lys, Arg or Met); Xaa at res. 4 = (His, Arg or Gln); and Xaa at res. 5 = (Glu, Ser, His, Gly, Arg, Pro, Thr, or Tyr).

25 In another embodiment, useful osteogenic proteins include those defined by Generic Sequences 9 and 10 (SEQ ID NOS: 6 and 7, respectively), described herein above. Specifically, Generic Sequences 9 and 10 are composite amino acid sequences of the following proteins: human OP-1, human OP-2, human OP-3, human BMP-2, human BMP-3, human BMP-4, human

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BMP-5, human BMP-6, human BMP-8, human BMP-9, human BMP-10, human BMP-11, *Drosophila* 60A, *Xenopus* Vg-1, sea urchin UNIVIN, human CDMP-1 (mouse GDF-5), human CDMP-2 (mouse GDF-6, human BMP-13), human CDMP-3 (mouse GDF-7, human BMP-12), mouse GDF-3, human GDF-1, mouse GDF-1, chicken DORSALIN, *Drosophila* dpp, *Drosophila* SCREW, mouse NODAL, mouse GDF-8, human GDF-8, mouse GDF-9, mouse GDF-10, human GDF-11, mouse GDF-11, human BMP-15, and rat BMP-3b. Like Generic Sequence 7, Generic Sequence 9 accommodates the C-terminal six cysteine skeleton and, like Generic Sequence 8, Generic Sequence 10 accommodates the seven cysteine skeleton.

**Generic Sequence 9 (SEQ ID NO: 6)**

Xaa 1	Xaa	Xaa	Xaa	Xaa 5	Xaa	Xaa	Xaa	Xaa	Xaa 10
Xaa	Xaa	Xaa	Xaa	Xaa 15	Xaa	Pro	Xaa	Xaa	Xaa 20
Xaa	Xaa	Xaa	Xaa	Cys 25	Xaa	Gly	Xaa	Cys	Xaa 30
Xaa	Xaa	Xaa	Xaa	Xaa 35	Xaa	Xaa	Xaa	Xaa	Xaa 40
Xaa	Xaa	Xaa	Xaa	Xaa 45	Xaa	Xaa	Xaa	Xaa	Xaa 50
Xaa	Xaa	Xaa	Xaa	Xaa 55	Xaa	Xaa	Xaa	Xaa	Xaa 60
Xaa	Cys	Xaa	Pro	Xaa 65	Xaa	Xaa	Xaa	Xaa	Xaa 70
Xaa	Xaa	Leu	Xaa	Xaa 75	Xaa	Xaa	Xaa	Xaa	Xaa 80
Xaa	Xaa	Xaa	Xaa	Xaa 85	Xaa	Xaa	Xaa	Xaa	Xaa 90
Xaa	Xaa	Xaa	Cys	Xaa 95	Cys	Xaa			

10 wherein each Xaa is independently selected from a group of one or more specified amino acids defined as follows: "Res. " means "residue" and Xaa at res. 1 = (Phe, Leu or Glu); Xaa at res. 2 = (Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu); Xaa at res. 3 = (Val, Ile, Leu or Asp); Xaa at res. 4 = (Ser, Asp, Glu, Asn or Phe); Xaa at res. 5 = (Phe or Glu); Xaa at res. 6 = (Arg, Gln, Lys, Ser, Glu, Ala or Asn); Xaa at res. 7 = (Asp, Glu, Leu, Ala or Gln); Xaa at res. 8 = (Leu, Val, Met, Ile or Phe); Xaa at res. 9 = (Gly, His or Lys); Xaa at res. 10 = (Trp or Met); Xaa at res. 11 = (Gln, Leu, His, Glu, Asn, Asp, Ser or Gly); Xaa at res. 12 = (Asp, Asn, Ser, Lys, Arg, Glu or His); Xaa at res. 13 = (Trp or Ser); Xaa at res. 14 = (Ile or Val); Xaa at res. 15 = (Ile or Val);

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Xaa at res. 16 = (Ala, Ser, Tyr or Trp); Xaa at res. 18 = (Glu, Lys, Gln, Met, Pro, Leu, Arg, His or Lys); Xaa at res. 19 = (Gly, Glu, Asp, Lys, Ser, Gln, Arg or Phe); Xaa at res. 20 = (Tyr or Phe); Xaa at res. 21 = (Ala, Ser, Gly, Met, Gln, His, Glu, Asp, Leu, Asn, Lys or Thr); Xaa at res. 22 = (Ala or Pro); Xaa at res. 23 = (Tyr, Phe, Asn, Ala or Arg); Xaa at res. 24 = (Tyr, His, Glu, Phe or Arg); Xaa at res. 26 = (Glu, Asp, Ala, Ser, Tyr, His, Lys, Arg, Gln or Gly); Xaa at res. 28 = (Glu, Asp, Leu, Val, Lys, Gly, Thr, Ala or Gln); Xaa at res. 30 = (Ala, Ser, Ile, Asn, Pro, Glu, Asp, Phe, Gln or Leu); Xaa at res. 31 = (Phe, Tyr, Leu, Asn, Gly or Arg); Xaa at res. 32 = (Pro, Ser, Ala or Val); Xaa at res. 33 = (Leu, Met, Glu, Phe or Val); Xaa at res. 34 = (Asn, Asp, Thr, Gly, Ala, Arg, Leu or Pro); Xaa at res. 35 = (Ser, Ala, Glu, Asp, Thr, Leu, Lys, Gln or His); Xaa at res. 36 = (Tyr, His, Cys, Ile, Arg, Asp, Asn, Lys, Ser, Glu or Gly); Xaa at res. 37 = (Met, Leu, Phe, Val, Gly or Tyr); Xaa at res. 38 = (Asn, Glu, Thr, Pro, Lys, His, Gly, Met, Val or Arg); Xaa at res. 39 = (Ala, Ser, Gly, Pro or Phe); Xaa at res. 40 = (Thr, Ser, Leu, Pro, His or Met); Xaa at res. 41 = (Asn, Lys, Val, Thr or Gln); Xaa at res. 42 = (His, Tyr or Lys); Xaa at res. 43 = (Ala, Thr, Leu or Tyr); Xaa at res. 44 = (Ile, Thr, Val, Phe, Tyr, Met or Pro); Xaa at res. 45 = (Val, Leu, Met, Ile or His); Xaa at res. 46 = (Gln, Arg or Thr); Xaa at res. 47 = (Thr, Ser, Ala, Asn or His); Xaa at res. 48 = (Leu, Asn or Ile); Xaa at res. 49 = (Val, Met, Leu, Pro or Ile); Xaa at res. 50 = (His, Asn, Arg, Lys, Tyr or Gln); Xaa at res. 51 = (Phe, Leu, Ser, Asn, Met, Ala, Arg, Glu, Gly or Gln); Xaa at res. 52 = (Ile, Met, Leu, Val, Lys, Gln, Ala or Tyr); Xaa at res. 53 = (Asn, Phe, Lys, Glu, Asp, Ala, Gln, Gly, Leu or Val); Xaa at res. 54 = (Pro, Asn, Ser, Val or Asp); Xaa at res. 55 = (Glu, Asp, Asn, Lys, Arg, Ser, Gly, Thr, Gln, Pro or His); Xaa at res. 56 = (Thr, His, Tyr, Ala, Ile, Lys, Asp, Ser, Gly or Arg); Xaa at res. 57 = (Val, Ile, Thr, Ala, Leu or Ser); Xaa at res. 58 = (Pro, Gly, Ser, Asp or Ala); Xaa at res. 59 = (Lys, Leu, Pro, Ala, Ser, Glu, Arg or Gly); Xaa at res. 60 = (Pro, Ala, Val, Thr or Ser); Xaa at res. 61 = (Cys, Val or Ser); Xaa at res. 63 = (Ala, Val or Thr); Xaa at res. 65 = (Thr, Ala, Glu, Val, Gly, Asp or Tyr); Xaa at res. 66 = (Gln, Lys, Glu, Arg or Val); Xaa at res. 67 = (Leu, Met, Thr or Tyr); Xaa at res. 68 = (Asn, Ser, Gly, Thr, Asp, Glu, Lys or Val); Xaa at res. 69 = (Ala, Pro, Gly or Ser); Xaa at res. 70 = (Ile, Thr, Leu or Val); Xaa at res. 71 = (Ser, Pro, Ala, Thr, Asn or Gly); Xaa at res. 2 = (Val, Ile, Leu or Met); Xaa at res. 74 = (Tyr, Phe, Arg, Thr, Tyr or Met); Xaa at res. 75 = (Phe, Tyr, His, Leu, Ile, Lys, Gln or Val); Xaa at res. 76 = (Asp, Leu, Asn or Glu); Xaa at res. 77 = (Asp, Ser, Arg, Asn, Glu, Ala, Lys, Gly or Pro); Xaa at res. 78 = (Ser, Asn, Asp, Tyr, Ala, Gly, Gln, Met, Glu, Asn or Lys); Xaa at res. 79 = (Ser, Asn, Glu, Asp, Val, Lys, Gly, Gln or

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Arg); Xaa at res. 80 = (Asn, Lys, Thr, Pro, Val, Ile, Arg, Ser or Gln); Xaa at res. 81 = (Val, Ile, Thr or Ala); Xaa at res. 82 = (Ile, Asn, Val, Leu, Tyr, Asp or Ala); Xaa at res. 83 = (Leu, Tyr, Lys or Ile); Xaa at res. 84 = (Lys, Arg, Asn, Tyr, Phe, Thr, Glu or Gly); Xaa at res. 85 = (Lys, Arg, His, Gln, Asn, Glu or Val); Xaa at res. 86 = (Tyr, His, Glu or Ile); Xaa at res. 87 = (Arg, Glu, Gln, Pro or Lys); Xaa at res. 88 = (Asn, Asp, Ala, Glu, Gly or Lys); Xaa at res. 89 = (Met or Ala); Xaa at res. 90 = (Val, Ile, Ala, Thr, Ser or Lys); Xaa at res. 91 = (Val or Ala); Xaa at res. 92 = (Arg, Lys, Gln, Asp, Glu, Val, Ala, Ser or Thr); Xaa at res. 93 = (Ala, Ser, Glu, Gly, Arg or Thr); Xaa at res. 95 = (Gly, Ala or Thr); Xaa at res. 97 = (His, Arg, Gly, Leu or Ser). Further, after res. 53 in rBMP-3b and mGDF-10 there is an Ile; after res. 54 in GDF-1 there is a T; after res. 54 in BMP-3 there is a V; after res. 78 in BMP-8 and Dorsalin there is a G; after res. 37 in hGDF-1 there is Pro, Gly, Gly, Pro.

Generic Sequence 10 (SEQ ID NO: 7) includes all of Generic Sequence 9 (SEQ ID NO: 6) and in addition includes the following sequence (SEQ ID NO: 9) at its N-terminus:

SEQ ID NO: 9

Cys      Xaa      Xaa      Xaa      Xaa  
1                                  5

15 Accordingly, beginning with residue 6, each "Xaa" in Generic Sequence 10 is a specified amino acid defined as for Generic Sequence 9, with the distinction that each residue number described for Generic Sequence 9 is shifted by five in Generic Sequence 10. Thus, "Xaa at res. 1 =( Tyr, Phe, His, Arg, Thr, Lys, Gln, Val or Glu)" in Generic Sequence 9 refers to Xaa at res. 6 in Generic Sequence 10. In Generic Sequence 10, Xaa at res. 2 = (Lys, Arg, Gln, Ser, His, Glu, Ala, or Cys); Xaa at res. 3 = (Lys, Arg, Met, Lys, Thr, Leu, Tyr, or Ala); Xaa at res. 4 = (His, Gln, Arg, Lys, Thr, Leu, Val, Pro, or Tyr); and Xaa at res. 5 = (Gln, Thr, His, Arg, Pro, Ser, Ala, Gln, Asn, Tyr, Lys, Asp, or Leu).

Based upon alignment of the naturally-occurring morphogens within the definition of Generic Sequence 10, it should be clear that gaps and/or insertions of one or more amino acid  
25 residues can be tolerated (without abrogating or substantially impairing biological activity) at least between or involving residues 11-12, 42-43, 59-60, 68-69 and 83-84.

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As noted above, certain currently preferred morphogenic polypeptide sequences useful in this invention have greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the preferred reference sequence of hOP-1. These particularly preferred sequences include allelic and phylogenetic counterpart variants of the OP-1 and OP-2 proteins, including the *Drosophila* 60A protein, as well as the closely related proteins BMP-5, BMP-6 and Vgr-1. Accordingly, in certain particularly preferred embodiments, useful morphogenic proteins include active proteins comprising pairs of polypeptide chains within the generic amino acid sequence herein referred to as "OPX" (SEQ ID NO: 3), which defines the seven cysteine skeleton and accommodates the homologies between several identified variants of OP-1 and OP-2. Accordingly, each "Xaa" at a given position in OPX independently is selected from the residues occurring at the corresponding position in the C-terminal sequence of mouse or human OP-1 or OP-2. Specifically, each "Xaa" is independently selected from a group of one or more specified amino acids as defined below:

15	Cys	Xaa	Xaa	His	Glu	Leu	Tyr	Val	Ser	Phe	Xaa	Asp	Leu	Gly	Trp
	1				5					10				15	
	Xaa	Asp	Trp	Xaa	Ile	Ala	Pro	Xaa	Gly	Tyr	Xaa	Ala	Tyr	Tyr	Cys
					20					25				30	
	Glu	Gly	Glu	Cys	Xaa	Phe	Pro	Leu	Xaa	Ser	Xaa	Met	Asn	Ala	Thr
					35					40				45	
20	Asn	His	Ala	Ile	Xaa	Gln	Xaa	Leu	Val	His	Xaa	Xaa	Xaa	Pro	Xaa
					50					55				60	
	Xaa	Val	Pro	Lys	Xaa	Cys	Cys	Ala	Pro	Thr	Xaa	Leu	Xaa	Ala	Xaa
					65					70				75	
	Ser	Val	Leu	Tyr	Xaa	Asp	Xaa	Ser	Xaa	Asn	Val	Ile	Leu	Xaa	Lys
25					80					85				90	
	Xaa	Arg	Asn	Met	Val	Val	Xaa	Ala	Cys	Gly	Cys	His			
					95					100					

wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 11 = (Arg or Gln); Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr); Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr or His); and Xaa at res. 97 = (Arg or Lys).

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In still another preferred embodiment, useful morphogenically-active proteins have polypeptide chains with amino acid sequences comprising a sequence encoded by a nucleic acid that hybridizes with DNA or RNA encoding reference morphogen sequences, *e.g.*, C-terminal sequences defining the conserved seven cysteine skeletons of OP-1, OP-2, BMP-2, BMP-4, BMP-5, BMP-6, 60A, GDF-3, GDF-5, GDF-6, GDF-7 and the like. As used herein, high stringency hybridization conditions are defined as hybridization according to known techniques in 40% formamide, 5 X SSPE, 5 X Denhardt's Solution, and 0.1% SDS at 37°C overnight, and washing in 0.1 X SSPE, 0.1% SDS at 50°C. Standard stringency conditions are well characterized in standard molecular biology cloning texts. See, for example, MOLECULAR CLONING A LABORATORY MANUAL, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA CLONING, Volumes I and II (D.N. Glover ed., 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed., 1984); NUCLEIC ACID HYBRIDIZATION (B. D. Hames & S.J. Higgins eds. 1984); and B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984).

In other embodiments, as an alternative to the administration of a morphogenic protein, an effective amount of an agent competent to stimulate or induce increased endogenous morphogen expression in a mammal may be administered by any of the routes described herein. Such a morphogen inducer may be provided to a mammal, *e.g.*, by systemic administration to the mammal or by direct administration to the neural tissue. A method for identifying and testing inducers (stimulating agents) competent to modulate the levels of endogenous morphogens in a given tissue is described in detail in published applications WO93/05172 and WO93/05751, the teachings of which are incorporated herein by reference. Briefly, candidate compounds can be identified and tested by incubation *in vitro* with a test tissue or cells thereof, or a cultured cell line derived therefrom, for a time sufficient to allow the compound to affect the production, *i.e.*, the expression and/or secretion, of a morphogen produced by the cells of that tissue. Suitable tissue, or cultured cells of a suitable tissue, preferably can be selected from renal epithelium, ovarian tissue, fibroblasts, and osteoblasts.

In yet other embodiments, an agent which acts as an agonist of a morphogen receptor may be administered instead of the morphogen itself. Such an agent may also be referred to as a morphogen "mimic," "mimetic," or "analog." Thus, for example, a small peptide or other

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molecule which can mimic the activity of a morphogen in binding to and activating the morphogen's receptor may be employed as an equivalent of the morphogen. Preferably the agonist is a full agonist, but partial morphogen receptor agonists may also be advantageously employed. Methods of identifying such agonists are known in the art and include assays for compounds which induce morphogen-mediated responses (*e.g.*, induction of differentiation of metanephric mesenchyme, induction of endochondral bone formation). For example, methods of identifying morphogen inducers or agonists of morphogen receptors may be found in U.S.S.N. 08/478,097 filed June 7, 1995 and U.S.S.N. 08/507,598 filed July 26, 1995, disclosures of which are incorporated herein by reference.

### 10           C.     Inhibition of Morphogen Activity

The inventors have found that endogenous morphogen-inhibitory compounds or exogenous morphogen inhibitors exist which inhibit morphogen activity. Such inhibition is generally related to the presence of cytokines, retinoids and/or cAMP, and related compounds. Cytokines such as LIF and CTNF inhibit morphogen-induced biological activity. Similarly, agents that increase intracellular cAMP (*e.g.*, dbcAMP, forskolin and neurotransmitters such as vasoactive intestinal polypeptide or "VIP") also decrease morphogen activity.

The methods of the present invention relate to potentiating endogenous morphogen activity by administering agents that inhibit or suppress the activity of the morphogen inhibitory compounds. Agents that release morphogen inhibition are appreciated by persons skilled in the art to be those that interfere with or suppress known morphogen-inhibitory signalling pathways and/or morphogen-inhibitory compounds. Morphogen-inhibition releasing agents can be any of numerous compounds such as polyclonal or monoclonal antibodies, analogs, enantiomers or other inhibitors known to inhibit or interfere with the activity of morphogen-inhibitory signalling pathways and/or morphogen-inhibitory compounds. For example, a monoclonal antibody to the human gp130 protein (the protein kinase which is part of the LIF receptor complex) suppresses LIF activity, and releases morphogen inhibition induced by LIF. Preferred agents that are capable of releasing morphogen inhibition include cytokine antagonists and retinoid antagonists. Preferred cytokine antagonists are neuropoietic cytokine antagonists, such as LIF antagonists or CTNF antagonists. Preferred retinoid antagonists include retinoic acid receptor antagonists and retinoid X receptor antagonists.

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Agents that interfere with cAMP signaling (i.e., cAMP dependent messenger pathways) are also capable of releasing morphogen inhibition. Particularly preferred are those agents that are inhibitors of protein kinase A. Such inhibitors include (2-p-bromocinnamylaminoethyl)-5-isoguinoline sulfonimide ("H89") and sterically constrained enantiomers of dibutyl cAMP and cAMP.

#### D. Formulations and Methods of Treatment

Compositions of the present invention (i.e., comprising a molecule capable of releasing morphogen inhibition, alone or in combination with a morphogen) may be administered by any route which is compatible with the particular molecule and, when included, with the particular morphogen. Thus, as appropriate, administration may be oral or parenteral, including intravenous and intraperitoneal routes of administration. In addition, administration may be by periodic injections of a bolus of the composition, or may be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an i.v. bag) or internal (e.g., a bioerodable implant, or a colony of implanted, morphogen-producing cells).

Therapeutic compositions of the present invention may be provided to an individual by any suitable means, directly (e.g., locally, as by injection, implantation or topical administration to a tissue locus) or systematically (e.g., parenterally or orally). Where the composition is to be provided parenterally, such as by intravenous, subcutaneous, intramolecular, ophthalmic, intraperitoneal, intramuscular, buccal, rectal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal or by aerosol administration, the composition preferably comprises part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the carrier or vehicle is physiologically acceptable so that in addition to delivery of the desired composition to the patient, it does not otherwise adversely affect the patient's electrolyte and/or volume balance. The fluid medium for the agent thus can comprise normal physiologic saline (e.g., 9.85% aqueous NaCl, 0.15M, pH 7-7.4).

For morphogens, association of the mature morphogen dimer with a morphogen pro domain results in the pro form of the morphogen which typically is more soluble in physiological solutions than the corresponding mature form. In fact, endogenous morphogens are thought to be transported (e.g., secreted and circulated) in the mammalian body in this form. This soluble form of the protein can be obtained from culture medium of morphogen-secreting mammalian cells,



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e.g., cells transfected with nucleic acid encoding and competent to express the morphogen. Alternatively, a soluble species can be formulated by complexing the mature, morphogenically-active polypeptide dimer (or an active fragment thereof) with a morphogen pro domain polypeptide or a solubility-enhancing fragment thereof. Solubility-enhancing pro domain fragments can be any N-terminal, C-terminal or internal fragment of the pro region of a member of the morphogen family that complexes with the mature polypeptide dimer to enhance stability and/or dissolubility of the resulting noncovalent or covalent complex. Typically, useful fragments are those cleaved at the proteolytic site Arg-Xaa-Xaa-Arg. A detailed description of soluble complex forms of morphogenic proteins, including how to make, test and use them, is described in WO 94/03600 (PCT US 93/07189). In the case of OP-1, useful pro domain polypeptide fragments include the intact pro domain polypeptide (residues 30-292) and fragments 48-292 and 158-292, all of SEQ ID NO: 2. Another molecule capable of enhancing solubility and particularly useful for oral administrations, is casein. For example, addition of 0.2% casein increases solubility of the mature active form of OP-1 by 80%. Other components found in milk and/or various serum proteins may also be useful.

In the context of the present invention, the morphogenic protein or peptide whose activity is released from inhibition may be endogenously expressed or exogenously provided. Further, DNA encoding the morphogenic protein or peptide may be incorporated into a recombinant vector and provided to the subject by well-known gene therapy methods.

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES (Gennaro, A., ed.), Mack Pub., 1990. Formulations of the therapeutic agents of the invention may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity to help maintain the agent at the desired locus. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, tricalcium phosphate, polybutyrate, lactide, and glycolide polymers and lactide/glycolide copolymers, may be useful excipients to control the release of the agent *in vivo*. Other potentially useful parenteral delivery systems for these agents include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and

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liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include  
5 glycocholate for buccal administration, methoxysalicylate for rectal administration, or cutric acid for vaginal administration. Suppositories for rectal administration may also be prepared by mixing the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) with a non-irritating excipient such as cocoa butter or other compositions which are solid at room temperature and liquid at body temperatures.

10 Formulations for topical administration to the skin surface may be prepared by dispersing the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) with a dermatologically acceptable carrier such as a lotion, cream, ointment or soap. Particularly useful are carriers capable of forming a film or layer over the skin to localize application and inhibit removal. For topical administration to internal tissue surfaces, the agent  
15 may be dispersed in a liquid tissue adhesive or other substance known to enhance adsorption to a tissue surface. For example, hydroxypropylcellulose or fibrinogen/thrombin solutions may be used to advantage. Alternatively, tissue-coating solutions, such as pectin-containing formulations may be used.

Where the composition is intended for use as a therapeutic for disorders of the CNS, an  
20 additional problem must be addressed: overcoming the blood-brain barrier, the brain capillary wall structure that effectively screens out all but selected categories of substances present in the blood, preventing their passage into the brain. The blood-brain barrier can be bypassed effectively by direct infusion of the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) into the brain, or by intranasal administration or inhalation of  
25 formulations suitable for uptake and retrograde transport by olfactory neurons.

#### **Example 1: Inhibition of Morphogen Activity**

##### ***1.1 Cytokine-Induced Inhibition of Morphogen-Induced Dendritic Growth***

Cytokines are a group of low molecular-weight regulatory proteins secreted by white blood cells and a variety of other cells in the body in response to a number of inducing stimuli,

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such as infections and tissue injury. Cytokines bind to specific receptors on the membrane of target cells, eliciting biochemical changes responsible for signal transduction that results in an altered pattern of gene expression in the target cells. These receptors are expressed by many types of cells and, as such, cytokines can affect a diverse array of cells. The binding of a cytokine to its  
5 receptor induces numerous physiologic responses including the development of cellular and humoral immune responses, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and induction of wound healing. For review see, e.g., IMMUNOLOGY, ch. 13 (2nd ed., Kuby, ed., W.H. Freeman and Company, 1994).

LIF and CNTF belong to the neurotrophic family of cytokines. Patterson and Nawa, *Cell*  
10 72: 123-137 (1993). Members of this family bind to receptors that exhibit various degrees of homology and form complexes with the gp130 signal transducing subunit. Ip and Yancopoulos, *Annu. Rev. Neurosci.* 19: 491-515 (1996). Receptors for neurotrophic cytokines are expressed on many types of cells in the central and peripheral nervous systems. Lo, *Proc. Natl. Acad. Sci. USA* 90: 2557-2558 (1993), and their activation regulates diverse aspects of neuronal development,  
15 including proliferation, Ernsberger, *et al.*, *Neuron* 2: 1275-1284 (1989), survival, Adler, *et al.*, *Science* 204: 1434-1436 (1979); Ip and Yancopoulos, *Annu. Rev. Neurosci.* 19: 491-515 (1996), and gene expression, Patterson and Nawa, *Cell* 72: 123-137 (1993).

Sympathetic neurons are among the cells that respond to LIF and CNTF, and this model system has been widely used to analyze the effects of these cytokines on gene expression. In  
20 addition, axotomy produces a large increase in LIF expression in peripheral nerve. Banner and Patterson, *Proc. Natl. Acad. Sci. USA* 91: 7109-7113 (1994).

Morphogens have been shown to enhance survival of neurons and maintain neural pathways at risk of injury, or following damage to nerve tissue. See International application publication WO 94/03200. Morphogens have also been shown to induce dendritic growth in  
25 sympathetic neurons. United States Patent Applications U.S.S.N. 08/292,782 and 08/926,154, the disclosures of which are incorporated by reference. Since sympathetic neurons are responsive to both morphogens and cytokines, the possibility was considered that cytokines might interact with morphogens in these cells. The neurite outgrowth model was used to assess this possibility.

Mature human recombinant OP-1 was isolated from medium conditioned by transfected  
30 Chinese hamster ovary cells using S-Sepharose and phenyl-Sepharose chromatography followed

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by reverse phase high performance liquid chromatography as described above. Other growth factors were obtained from commercial sources: Recombinant human LIF, interleukin-11, transforming growth factors  $\beta$ -1, and affinity-purified goat immunoglobulin to human LIF from R&D Systems (Minneapolis, MN); CNTF and GDNF from PeproTech, Inc. (Rocky Hill, NJ);  
5 epidermal growth factor, basic fibroblast growth factor, and interleukin-6 from Life Technologies (Gaithersburg, MD); and platelet derived growth factor from Collaborative Biomedical Products (Bedford, MA).

Suspensions of neurons dissociated from the superior cervical ganglia of Sprague-Dawley rat fetuses (19-21 day) or rat pups (1-3 day postnatal) were prepared according to the method of  
10 Higgins, *et al.*, CULTURING NERVE CELLS, Banker and Goslin, eds., MIT Press, pp. 177-205 (1991), the teachings of which herein incorporated by reference. Equivalent results were obtained with pre- and postnatal animals. Neurons were plated at low density (about 15 cells/mm<sup>2</sup>) onto poly-D-lysine coated coverslips and maintained in a serum-free medium containing NGF (100 ng/ml) Higgins, *et al.*, CULTURING NERVE CELLS (1991). Cytosine-b-D-furanoside (1  $\mu$ M) was  
15 added to the medium of all cultures for 48 hrs on the second day. This exposure was sufficient to render them virtually free of nonneuronal cells for 30 days. Experimental treatments were started on the fifth day, after non-neuronal cells had been eliminated by exposure to an antimitotic agent.

Cellular morphology was routinely assessed by intracellular injection of fluorescent dyes (4% Lucifer Yellow or 5% 5,6 dicarboxyfluorescein; Bruckenstein and Higgins, *Dev. Biol.* 128:  
20 924-936 (1988). Only neurons whose cell bodies were at least 150  $\mu$ m from their nearest neighbor were injected, because density-dependent changes in morphology occur when somata of sympathetic neurons are separated by lesser distances.

LIF stimulated dendritic growth. When compared to OP-1 induced dendritic growth, marked differences in both the time course and the magnitude of the response to these agents  
25 were observed (FIG. 2). The response to OP-1 was rapid and robust. Thus, large increases in dendritic growth were apparent after only 24 hr of treatment, and neurons eventually formed an average of approximately 6 primary dendrites/cell in the presence of this trophic factor. In contrast, a 10 day exposure to LIF was required to elicit detectable changes in the percentage of cells with dendrites (FIG. 2). In addition, it was found that a smaller percentage of cells  
30 responded to LIF (50% after 10 day of treatment) as compared to OP-1 (100%) and that the cells

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treated with LIF had fewer and shorter dendrites. Thus, the net result of a 15 d exposure to LIF was the production of a subpopulation of cells which typically has 1 or 2 short unbranched dendrites. Longer exposures (4-6 weeks) to LIF failed to significantly increase either the number or the length of the primary dendrites (data not shown).

5           In order to assess possible synergistic effects between morphogens and cytokines, sympathetic neurons were exposed to both LIF and OP-1. Instead of an additive effect, neurons that had been treated with both LIF and OP-1 closely resembled neurons that had been exposed to LIF alone in terms of the percentage of neurons with dendrites, the number of dendrites/cell and the length of the longest dendrite (FIG. 2). These data suggest that LIF has two different and  
10       opposing effects on cellular morphology: it not only weakly stimulates dendritic growth by itself, but also profoundly depresses the morphogen-induced dendritic growth. The inhibitory effects of LIF were still apparent after 15 day of treatment (FIG. 2), indicating that they were long-lasting. Moreover, the inhibitory effect was obtained when added in conjunction with OP-1 at day 3 or subsequently at day 5 or 7 (FIG. 3).

15           CNTF, another cytokine, also stimulated basal dendritic growth and inhibited the response to OP-1 (FIG. 4). Moreover, the magnitude of the effects and the range of effective doses were similar to those observed with LIF. In contrast, neither stimulatory nor inhibitory effects on dendritic growth were observed with other neuropoietic cytokines such as interleukin-6 and interleukin-11 or with several unrelated growth factors (Table I).

20           Examination of concentration-effect curves revealed that stimulation of basal dendritic growth and inhibition of OP-1-induced dendritic growth could both be consistently obtained with concentrations of LIF or CTNF as low as 1 ng/ml (FIG. 3). These data suggested that both phenomena reflected a specific effect of the recombinant protein, and this was confirmed by antibody inhibition experiments. Thus, both the stimulatory effects of LIF on basal dendritic  
25       growth (not shown) and the inhibitory effects of LIF on OP-1-induced dendritic growth (FIG. 5) were substantially reduced when 10-30  $\mu$ g/ml of an affinity purified polyclonal anti-LIF antibody was added to the medium. These data suggest that both types of changes in dendritic growth represent specific responses to the recombinant protein and not to contaminants.

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TABLE I

**EFFECTS OF VARIOUS GROWTH FACTORS ON BASAL  
AND OP-1-STIMULATED DENDRITIC GROWTH**

<b>Growth Factor (ng/ml)</b>	<b>Growth Factor Alone (dendrites/cell)</b>	<b>Growth Factor with OP-1 (dendrites/cell)</b>
Control	0.17 ± 0.07	6.50 ± 0.24
LIF (30)	1.00 ± 0.21	1.13 ± 0.23*
CNTF (30)	0.97 ± 0.21	1.83 ± 0.28*
IL-6(30)	0.13 ± 0.06	6.37 ± 0.31
IL-11(30)	0.40 ± 0.11	6.00 ± 0.23
TGFβ1 (20)	0.20 ± 0.09	6.27 ± 0.31
GDNF (50)	0.13 ± 0.06	6.47 ± 0.29
EGF (30)	0.17 ± 0.08	6.07 ± 0.22
βFGF (30)	0.17 ± 0.08	6.00 ± 0.27
PDGF (100)	0.23 ± 0.08	6.37 ± 0.31

Treatments started five days after glia were eliminated. The number of dendrites/ cells was measured by dye injection after 10 days of exposure to various growth factors. Data are expressed as mean ± SEM. N = 30. \*p < 0.001 vs control.

These observations suggest that certain cytokines can inhibit morphogen activity in sympathetic neurons, even in the presence of optimal concentrations of morphogens. Since cytokines are secreted in response to stimuli such as infections and tissue injury, the inhibitory effects of endogenously-released cytokines may compromise the ability of an endogenous or exogenous morphogen to replace diseased or damaged tissues.

### ***1.2 Retinoid-Induced Inhibition of Morphogen-Induced Dendritic Growth***

Retinoids, a class of compounds including retinol (vitamin A), retinoic acid (RA), and a series of natural and synthetic derivatives, exhibit striking effects on cell proliferation, differentiation, and pattern formation during development. Strickland, *et al.*, *Cell*, 15: 393-403 (1978); Breitman, *et al.*, *Proc: Natl. Acad. Sci. USA*, 77: 2936-2940 (1980); Roberts, *et al.*, *THE RETINOIDS*, Vol. 2 eds. (1984); Thaller, *et al.*, *Nature*, 327: 625-628 (1987). Retinoids modify their target cells through specific receptors. See U.S. Patent No. 5,317,090; Giguere, *et al.*, *Nature*, 330: 624-629 (1987). Since sympathetic neurons have retinoid receptors and retinoids have been demonstrated to exhibit striking effects on cell proliferation, differentiation, and pattern

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formation during development, the model described in Example 1.1 was used to investigate the effects of these agents on basal and OP-1 stimulated neuronal growth.

Suspensions of neurons dissociated from the superior cervical ganglia of Sprague-Dawley rat embryos (17 day) were prepared according to the method of Higgins, *et al.*, CULTURING  
5 NERVE CELLS, Banker and Goslin, eds., MIT Press, pp. 177-205 (1991), the teachings of which herein incorporated by reference. Neurons were plated at low density (about 15 cells/mm<sup>2</sup>) onto poly-D-lysine coated coverslips and maintained in a serum-free medium containing NGF (100 ng/ml) Higgins, *et al.*, CULTURING NERVE CELLS (1991). Cytosine-b-D-furanoside (1  $\mu$ M) was added to the medium of all cultures for 48 hrs on the second day. This exposure was sufficient to  
10 render them virtually free of nonneuronal cells for 30 days. Experimental treatments were started on the fifth day, after non-neuronal cells had been eliminated by exposure to an antimitotic agent.

Axons were identified with a rabbit polyclonal antiserum to the 200 kDa neurofilament protein (NF-H; Sigma; 1:100, 91  $\mu$ g/ml). A monoclonal antibody to microtubule-associated protein 2 (MAP2; Boehringer-Mannheim; 1:100, 20  $\mu$ g/ml) was used as a specific marker of  
15 dendrites. To visualize these intracellular antigens, cells were permeabilized with 0.5% triton X-100 (TX) in 0.1 M PBS containing 1% BSA and 4% goat serum (GS; Sigma) for 1 hr at 25°C. Primary antibodies were diluted in 0.1M PBS, 1% BSA, 4% GS with 0.5% TX and incubated for 1 hr at 37°C. After the primary incubation, the cells were washed three times in PBS containing 4% GS. Labeling was detected with fluorescein- or rhodamine-conjugated antibodies (1:400 in  
20 PBS, BSA, GS, and TX, 1 hr at 37°C, in the dark). Mouse antibodies were visualized with fluorescein-coupled goat anti-mouse Ig (Boehringer-Mannheim). Rabbit or rat antibodies were visualized using indirect immunofluorescence with rhodamine-conjugated goat anti-rat or anti-rabbit Ig. Cultures were additionally stained with a nuclear stain, 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma; 0.1  $\mu$ g/ml, 5 min at room temperature).  
25 Coverslips were washed once in sterile water and let dry for 10 min before mounting onto glass slides in aqueous mounting solution (Fluoromount; Southern Biotechnology). Slides were kept refrigerated in the dark until examined.

Retinoic acid did not stimulate dendritic growth. The number and length of dendrites were not significantly increased by exposure to 1 nM to 3  $\mu$ M retinoic acid (Table II; FIG. 6).  
30 However, when sympathetic neurons were exposed to both retinoic acid and OP-1, retinoic acid

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profoundly depressed the morphogen-induced dendritic growth. Examination of concentration-effect curves revealed that inhibition of OP-1-induced dendritic growth to be dose-dependent and could be consistently obtained with concentrations of retinoic acid as low as 1 nM (Table II; FIG. 6; see also FIG. 7). As summarized in Table II, retinoic acid decreased OP-1-induced increases in dendrite numbers, length of the longest dendrite, and total length of dendrite.

**TABLE II**  
**EFFECTS OF RETINOIC ACID ON BASAL**  
**AND OP-1-STIMULATED DENDRITIC GROWTH**

Condition	# of dendrites	Length of longest dendrite ( $\mu$ m)	Total length of dendrite ( $\mu$ m)
Control	$0.03 \pm 0.03$ (30)	$25 \pm 0$ (1)	$25 \pm 0$ (1)
DMSO	$0.02 \pm 0.02$ (30)	$54.65 \pm 0$ (1)	$54.65 \pm 0$ (1)
DMSO + 50 ng/ml OP-1	$2.20 \pm 0.21$ (30)	$72.11 \pm 6.77$ (29)	$128.17 \pm 13.20$ (29)
1 nM RA	$0.03 \pm 0.03$ (30)	$31.83 \pm 0$ (1)	$31.83 \pm 0$ (1)
3 nM RA	$0.02 \pm 0.02$ (30)	$21.59 \pm 0$ (1)	$21.59 \pm 0$ (1)
10 nM RA	$0 \pm 0$ (30)	$0 \pm 0$ (0)	$0 \pm 0$ (0)
300 nM RA	$0.03 \pm 0.03$ (30)	$44.64 \pm 0$ (1)	$44.64 \pm 0$ (1)
3 $\mu$ M RA	$0.1 \pm 0.06$ (30)	$51.52 \pm 24.05$ (3)	$51.52 \pm 24.05$ (3)
50 ng/ml OP-1	$2.13 \pm 0.21$ (30)	$91.63 \pm 6.79$ (27)	$153.53 \pm 12.04$
1 nM RA + 50 ng/ml OP-1	$1.77 \pm 0.19$ (30)	$74.40 \pm 8.49$ (27)	$115.68 \pm 11.91$ (27)
3 nM RA + 50 ng/ml OP-1	$1.33 \pm 0.17$ (30)	$70.33 \pm 6.92$ (25)	$100.51 \pm 12.46$ (25)
10 nM RA + 50 ng/ml OP-1	$0.8 \pm 0.19$ (30)	$53.04 \pm 6.84$ (14)	$82.92 \pm 14.13$ (14)
100 nM RA + 50 ng/ml OP-1	$0.77 \pm 0.14$ (30)	$50.58 \pm 6.39$ (17)	$61.14 \pm 6.37$ (17)
300 nM RA + 50 ng/ml OP-1	$0.57 \pm 0.16$ (30)	$49.81 \pm 4.89$ (11)	$70.16 \pm 11.89$ (11)
3 $\mu$ M RA + 50 ng/ml OP-1	$0.43 \pm 0.11$ (30)	$37.44 \pm 3.13$ (11)	$42.43 \pm 5.01$ (11)

These observations suggest that certain retinoids can inhibit morphogenic activity, even in the presence of optimal concentrations of morphogens. Thus, the inhibitory effects of retinoids may compromise the ability of an endogenous or exogenous morphogen to replace diseased or damaged tissues.

### ***1.3 Inhibition of Morphogen-Induced Dendritic Growth by Agents Affecting cAMP***

Agents that increase intracellular cyclic AMP have been found to decrease OP-1 induced dendritic growth. These agents include dbcAMP, forskolin and neurotransmitters such as VIP.

Cultures of rat sympathetic neurons were treated for 5 days with OP-1 (50ng/ml) alone or OP-1 plus various concentrations of either forskolin or db-cAMP. Cellular morphology was then



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analyzed by microinjection of fluorescent dye. Results are shown in FIG 8. Both the number of dendrites per cell and the maximum extension of the dendritic arbors were decreased in a concentration-dependent manner by forskolin and db-cAMP. Mean  $\pm$  S.E.M. (N=30).

Neural injury can lead to increased synthesis of transmitters that increase cAMP, and these agents may cause dendritic retraction in vivo. Accordingly, drugs that interfere with cAMP signaling by inhibiting protein kinase A can act to increase OP-1 dendritic growth. Such protein kinase A inhibitors include H89 ((2-p-bromocinnamylaminoethyl)-5-isoquinolinesulfonamide) and sterically constrained enantiomers of cAMP and dibutyl cAMP.

#### **Example 2: Molecules Capable of Releasing the Inhibition of Morphogen Activity**

As described in Example 1, various factors have been shown to inhibit morphogen activity in mammalian cells. These inhibitors can compromise the ability of endogenous or exogenous morphogen to replace diseased or damaged tissues.

According to the present invention, morphogen activity is potentiated by the administration of a molecule capable of antagonizing the inhibitory effects of these factors. These antagonists are any molecules that inhibit the biological activity of a factor that inhibits morphogen activity in mammalian cells. The molecules antagonize the effects of the inhibitory factors by, for example, (1) binding directly to the receptor of the inhibitory factor but fail to activate the receptor signal transduction element, thereby making the receptor unavailable for binding with the inhibitory factor; (2) binding directly to the inhibitory factor, thereby preventing the inhibitor from interacting with the receptor; (3) inhibiting signal transduction once the inhibitory factor has bound directly to the receptor, thereby neutralizing the biological effects of the inhibitory factor. The antagonists are inorganic molecules or organic molecules (*e.g.*, protein, peptides).

As described in Example 1, neuropoietic cytokines have been shown to inhibit morphogen activity in mammalian cells. The inhibitory effects of these endogenously-released cytokines were observed in the presence of optimal concentrations of the morphogen.

LIF and CNTF belong to the neuropoietic family of cytokines. Patterson and Nawa, *Cell* 72: 123-137 (1993). Members of this family bind to receptors that exhibit various degrees of homology and form complexes with the gp130 signal transducing subunit. Ip and Yancopoulos,

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*Annu. Rev. Neurosci.* 19: 491-515 (1996). According to the present invention, morphogen activity are potentiated by the administration of a cytokine antagonist.

A number of protein that inhibit the biological activity of cytokines have been reported. See, e.g., IMMUNOLOGY, ch. 13 (2nd ed., Kuby, ed., W.H. Freeman and Company, 1994).

- 5 Cytokine antagonists can antagonize the inhibitory effects of cytokines by, for example, (1) binding directly to the cytokine receptor but fail to activate the cell, thereby making the receptor unavailable for binding cytokines; (2) binding directly to the cytokine, thereby preventing the inhibitor from interacting with the receptor; (3) inhibiting signal transduction once the cytokine has bound directly to the receptor. Examples of the second group of cytokine
- 10 antagonists include soluble cytokine receptors that are able to bind to the cytokine and neutralize its activity. Enzymatic cleavage of the extracellular domain of a cytokine receptor releases a soluble fragment that retains its cytokine-binding properties. Viruses have also been shown to produce cytokine-binding proteins. The poxviruses, for example, have been shown to encode a soluble TNF-binding protein and a soluble IL-1-binding protein. IMMUNOLOGY, ch. 13 (2nd ed.,
- 15 Kuby, ed., W.H. Freeman and Company, 1994).

- Methods described in Example 1.1 are used to assess the effects of cytokine antagonists on the cytokine-induced inhibition of morphogenic activity. Briefly, sympathetic neurons are exposed to OP-1 alone, OP-1 and LIF, or OP-1, LIF, and an LIF antagonist. Neurons treated with OP-1, LIF, and an LIF antagonist are expected to resemble neurons treated with OP-1 alone
- 20 in terms of the percentage of neurons with dendrites, the number of dendrites/cell and the length of the longest dendrite. These neurons exhibit significant increases in the number of dendrites/cell and the length of the longest dendrite when compared to neurons treated with OP-1 and LIF. Thus, the LIF antagonist is expected to release the morphogen inhibition induced by LIF.

### ***2.1 Antibody to gp 130 Reduces LIF-Induced Dendritic Retraction***

- 25 As described in Example 1, LIF inhibits dendritic growth in cultured sympathetic neurons. Because LIF is known to be released at the site of neural injury, it may mediate the dendritic retraction that typically follows axonal damage in vivo. Experiments were performed in which sympathetic neurons were exposed to a monoclonal antibody to the human gp130 protein, which is the protein kinase that is part of the LIF receptor complex.

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Cultures of sympathetic neurons were treated with OP-1 (100 ng/ml) for 5 days to induce dendritic growth. Beginning on the sixth day, LIF (500 pg/ml), antibody to gp130 (MAB 228, 100 ug/ml), or both agents were added to the OP-1 containing medium of some cultures. Cellular morphology was assessed on the seventh day following immunostaining with antibody to nonphosphorylated forms of the M and H neurofilament subunits. Results are shown in Table III, below. The concentration of LIF used in this experiment produced a 39% decrease in the size of the dendritic arbor. A submaximal concentration was used because the interaction between MAB228 and gp130 is influenced by the concentration of the ligand and so inhibition of the LIF response was more apparent with concentrations near the ED<sub>50</sub> as compared with higher concentrations.

**TABLE III**  
**EFFECTS OF ANTIBODY TO gp130 ON LIF-INDUCED DENDRITIC RETRACTION**

Growth Factor	Dendrites/cell
OP-1	4.2 ± 0.3
OP-1 + LIF	2.6 ± 0.3
OP-1 + LIF + anti gp130	3.5 ± 0.4
OP-1 + anti gp130	4.2 ± 0.3

\* P<0.05 compared with OP-1+LIF treatment.

Thus, antibody to the gp130 protein reduces the response to LIF and enhances OP-1 induced dendritic growth.

## ***2.2 PI-PLC Reduces CNTF-Induced Dendritic Retraction***

The CNTF receptor complex is a heterotrimer consisting of gp130, the LIF receptor (LIFR) and CNTF receptor  $\alpha$  subunit (CNTFR) (Ip and Yancopoulos, 1996; Segal and Greenberg, 1996). The CNTFR specifically confers CNTF responsiveness and it is linked to the cell membrane via a glycosyl phosphatidylinositol bond which can be cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC). In contrast, the two subunits (gp130 and LIFR) that are required for responsiveness to LIF are integral membrane proteins which are unaffected by this enzyme. An examination of the effects of PI-PLC treatment on the response of cells to these cytokines revealed that CNTF-induced dendritic retraction was reduced by prior PI-PLC treatment while the response LIF remained intact (FIG. 9).

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Cultures of sympathetic neurons were exposed to OP-1 (50 ng/ml) for 3 days. Subsequently, some cultures were exposed for additional 2 days to OP-1 combined with CNTF (30 ng/ml) or LIF (30 ng/ml). Other cultures were treated with PI-PLC (1 U/ml) for 1 hour before receiving the aforementioned CNTF or LIF treatments. Cellular morphology was analyzed after immunostaining with a MAb to the nonphosphorylated forms of the H and M neurofilament subunits. Mean  $\pm$  S.E.M. (N=30). \*P < 0.01 vs OP + CNTF.

These results indicate that CNTF-induced dendritic retraction requires the intact CNTF receptor complex and that inhibition can be released by treatment with compounds that disrupt or cleave the CNTF receptor complex.

### 10    **Example 3    Screening Assays for Compounds Which Release the Inhibition of Morphogen Activity**

Compositions of the present invention have utility in any application where tissue morphogenesis is desired, such as in the regeneration of damaged tissue resulting from mechanical or chemical trauma, degenerative diseases, tissue destruction resulting from chronic inflammation, cirrhosis, inflammatory diseases, cancer and the like, and in the regeneration of tissues, organs and limbs.

Candidate compounds that will antagonize the inhibitory effects of inhibitor by binding directly to the receptor of the inhibitor (*e.g.*, cytokine and retinoid receptor antagonists) are initially screened for their ability to compete for binding at the receptor with the inhibitor, using competitive binding studies well known in the art. Candidate compounds with binding specificity for the receptor are further evaluated for their ability to inhibit the biological activity of the inhibitor.

Candidate compounds are evaluated for their ability to inhibit the biological activity of the inhibitor and to release morphogen inhibition by monitoring their effect on an appropriate morphogen responsive cell line. Cell cultures of kidney, liver, adrenals, urinary bladder, brain, or other organs, are prepared as described widely in the literature. For example, kidneys are explanted from neonatal or new born or young or adult rodents (mouse or rat) and used in organ culture as whole or sliced (1-4 mm) tissues. Primary tissue cultures and established cell lines, also derived from kidney, adrenals, urinary, bladder, brain, mammary, or other tissues can be established in multiwell plates (6 well or 24 well) according to conventional cell culture

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techniques, and are cultured in the absence or presence of serum for a period of time (1-7 days). Cells are cultured, for example, in DMEM containing 10% fetal calf serum or in serum-deprived medium or in defined medium (*e.g.*, containing insulin, transferrin, glucose, albumin, or other growth factors).

5           **3.1    Screening Assays for Compounds Which Release the Inhibition of Morphogen-Induced Dendritic Growth**

Candidate compounds are evaluated for their ability to release morphogen inhibition by monitoring their effect on dendritic growth. OP-1 has been demonstrated to induce dendritic growth in a variety of neuronal cells, including sympathetic neurons, hippocampal neurons,  
10   cerebral cortical neurons, spinal motor neurons, and mesencephalic neurons. See U.S. Patent Application Serial Nos. 08/938,622, 08/958,463 and 08/937,755, the disclosures of which are incorporated herein by reference.

Candidate compounds are evaluated essentially as described in Example 1.2. Briefly, dissociated neurons are exposed *in vitro* to (1) OP-1 alone, (2) OP-1 and the inhibitor, or  
15   (3) OP-1, the inhibitor, and various concentrations of a candidate compound. Briefly, pregnant Balb/c mice (E18) are euthanized by decapitation following CO<sub>2</sub> anesthesia and the embryos removed under sterile conditions. After carefully removing the meninges, the neuronal tissue is dissected in sterile Hank's balanced salt solution (HBSS) without Ca<sup>2+</sup>/Mg<sup>2+</sup> (Biowhittaker) containing 0.6% glucose and 0.5% HEPES (Sigma) and dissociated into a single-cell suspension.  
20   The neuronal tissue is minced to 1 mm thick pieces and dissociated into a single-cell suspension using the following protocol. Pieces are placed in 4.5 ml of Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS in a 50 ml conical culture tube and incubated in a water bath for 5 min at 37°C. 0.5 ml of 2.5% trypsin solution (Gibco) is added and the tissue is then incubated for 10 min on a shaking device at 37°C. The supernatant is then removed and placed into another tube containing 0.5 ml fetal bovine  
25   serum (FBS; Gibco). Five ml of 0.025% Deoxyribonuclease I (Dnase; Calbiochem Corp.) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS is then added to the pellet and the incubation is continued for another 5 min on a shaking device at 37°C. At the end of incubation, the trypsin is inactivated by adding 0.5 ml FBS. The supernatant collected earlier is combined with the tissue and the cells are then concentrated by centrifugation (1000 rpm, 5 min) and the supernatant is decanted. Fresh medium  
30   (2 ml) is added to resuspend the pellet which is further dissociated into a single-cell suspension by trituration using a pipet-tip.

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Neurons are plated in Neurobasal Medium (GIBCO), supplemented with B-27 Supplement (GIBCO), 1 mM glutamine and penicillin/streptomycin are plated at low density ( $1 \times 10^4$  cells/0.5 ml) on poly-D-lysine coated coverslips inserted into 24-well culture plate (Falcon). Cells are grown for two days *in vitro* at 37°C in an atmosphere of 5% CO<sub>2</sub>. OP-1 (1, 10, 30, or 100 ng/ml) is added either three hours or 24 hours after plating the mesencephalic neurons. BSA is added to all wells at a final concentration of 500 µg/ml prior to adding OP-1. Control cultures consists of culture medium and medium with BSA 500 µg/ml.

Mouse neurites are immunostained with M6, a mouse neuron-specific monoclonal antibody (donated by Dr. C. Laquaur, University of Pittsburgh). Cells are first incubated in 0.1 M phosphate buffered saline (PBS) containing 1% BSA for 30 min at 25°C and then exposed to M6 in PBS (1:10) for 24 hrs at 4°C. Immunofluorescent labeling for M6 is carried out using biotinylated secondary antibodies anti-rat IgG (Sigma; 3 µg/ml, 1:200, 1 hr at 37°C) followed by avidin-TRITC conjugate (Sigma; 6.5 µg/ml, 1:400, 1 hr at 37°C, in the dark).

Axons are identified with a rabbit polyclonal antiserum to the 200 kDa neurofilament protein (NF-H; Sigma; 1:100, 91 µg/ml). A monoclonal antibody to microtubule-associated protein 2 (MAP2; Boehringer-Mannheim; 1:100, 20 µg/ml) is used as a specific marker of dendrites. To visualize these intracellular antigens, cells are permeabilized with 0.5% Triton X-100 (TX) in 0.1 M PBS containing 1% BSA and 4% goat serum (GS; Sigma) for 1 hr at 25°C. Primary antibodies are diluted in 0.1 M PBS, 1% BSA, 4% GS with 0.5% TX and incubated for 1 hr at 37°C. After the primary incubation, the cells are washed three times in PBS containing 4% GS. Labeling is detected with fluorescein- or rhodamine-conjugated antibodies (1:400 in PBS, BSA, GS, and TX, 1 hr at 37°C, in the dark). Mouse antibodies are visualized with fluorescein-coupled goat anti-mouse Ig (Boehringer-Mannheim). Rabbit or rat antibodies are visualized using indirect immunofluorescence with rhodamine-conjugated goat anti-rat or anti-rabbit Ig. Cultures are additionally stained with a nuclear stain, 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma; 0.1 µg/ml, 5 min at room temperature). Coverslips are washed once in sterile water and allowed to dry for 10 min before mounting onto glass slides in aqueous mounting solution (Fluoromount; Southern Biotechnology). Slides are kept refrigerated in the dark until examined.

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Only isolated neurons whose cell bodies or processes that are not in contact with other neurons are analyzed. A total of 100 neurons are examined for each experimental condition. For measurements of neurite length, neurons are examined at a final image magnification of 400X. Fluorescent images of the neurons are recorded with a CCD video camera (Dage) and analyzed  
5 with a Macintosh PowerMac (9500/200) and image processing program (NIH Image 1.59). Neurite lengths are measured by tracing the total length of any neurite extending from a neuron cell body. Recorded lengths are calibrated at the same magnification using a ciroscope slide micrometer. Analysis of statistical significance of any observed differences between monolayers was performed using Student's *t*-test or ANOVA (SPSS/Mac, version 6.1, SPSS Inc., Chicago,  
10 IL).

Neurons treated with OP-1 alone and with OP-1, the inhibitor, and a molecule capable of releasing morphogen inhibition have significantly enhanced dendritic growth when compared to neurons treated with OP-1 and the inhibitor, reflected in increased length, diameter, and number of processes.

15       **3.2 Additional Screening Assays for Compounds Which Release the Inhibition of Morphogen Activity**

Candidate compounds can also evaluated by their ability to induce differentiation of primary osteoblasts, by measuring the ability of the potential analog to induce production of alkaline phosphatase, PTH-mediated cAMP and osteocalcin, all of which are induced when  
20 primary osteoblasts are exposed to OP-1, 60A or DPP.

In this example involving osteoblast cultures, rat osteoblast-enriched primary cultures are preferably used. Although these cultures are heterogeneous in that the individual cells are at different stages of differentiation, the culture is believed to reflect more accurately the metabolism and function of osteoblasts *in vivo* than osteoblast cultures obtained from established cell lines.  
25 (Unless otherwise indicated, all chemicals referenced are standard, commercially available reagents, readily available from a number of sources, including Sigma Chemical, Co., St. Louis; Calbiochem, Corp., San Diego and Aldrich Chemical Co., Milwaukee.)

Rat osteoblast-enriched primary cultures are prepared by sequential collagenase digestion of newborn suture-free rat calvaria (*e.g.*, from 1-2 day-old animals, Long-Evans strain, Charles  
30 River Laboratories, Wilmington, MA), following standard procedures, such as are described, for

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example, in Wong *et al.*, (1975) *PNAS* 72:3167-3171. Rat osteoblast single cell suspensions are then plated onto a multi-well plate (*e.g.*, a 24 well plate) at a concentration of 50,000 osteoblasts per well in alpha MEM (modified Eagle's medium, Gibco, Inc., Long Island) containing 10% FBS (fetal bovine serum), L-glutamine and penicillin/streptomycin. The cells are incubated for  
5 24 hours at 37°C, at which time the growth medium is replaced with alpha MEM containing 1% FBS and the cells incubated for an additional 24 hours so that the cells are in serum-deprived growth medium at the time of the experiment.

#### A. *Alkaline Phosphatase Induction in Osteoblasts*

Candidate compounds are evaluated for their ability to release morphogen inhibition by  
10 monitoring their effect on alkaline phosphatase induction in osteoblasts. The cultured cells in serum-free medium are incubated with (1) OP-1 alone (*e.g.*, 0.1, 1.0, 10.0, 40.0 or 80.0 ng/ml), (2) OP-1 and the inhibitor, or (3) OP-1, the inhibitor, and various concentrations of a candidate compound. 72 hours after the incubation period the cell layer is extracted with 0.5 ml of 1% Triton X-100. The resultant cell extract then, is centrifuged, and 100 ml of the extract is added to  
15 90 ml of paranitrosophenylphosphate (PNPP)/glycerine mixture and incubated for 30 minutes in a 37°C water bath and the reaction stopped with 100 ml NaOH. The samples are then run through a plate reader (*e.g.*, Dynatech MR700 plate reader, and absorbance measured at 400 nm, using p-nitrophenol as a standard) to determine the presence and amount of alkaline phosphate activity. Protein concentrations are determined by the Biorad method. Alkaline phosphatase activity is  
20 calculated in units/mg protein, where 1 unit=1 nmol p-nitrophenol liberated/30 minutes at 37°C. Cells treated with OP-1 alone and with OP-1, the inhibitor, and a molecule capable of releasing morphogen inhibition have a significantly higher specific activity of alkaline phosphate compared to cells treated with OP-1 and the inhibitor.

#### B. *Induction of PTH-Mediated cAMP*

Candidate compounds are evaluated for their ability to release morphogen inhibition by  
25 monitoring their effect on parathyroid hormone-mediated cAMP production in rat osteoblasts *in vitro*. Rat osteoblasts are prepared and cultured in a multiwell plate as described above. The cultured cells in serum-free medium are incubated with (1) OP-1 alone (*e.g.*, 0.1, 1.0, 10.0, 40.0 or 80.0 ng/ml), (2) OP-1 and the inhibitor, or (3) OP-1, the inhibitor, and various concentrations  
30 of a candidate compound. The plate is then incubated for another 72 hours. At the end of the



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72 hours the cells are treated with medium containing 0.5% bovine serum albumin (BSA) and 1mM 3-isobutyl-1-methylxanthine for 20 minutes followed by the addition into half of the wells of human recombinant parathyroid hormone (hPTH, Sigma, St. Louis) at a concentration of 200 ng/ml for 10 minutes. The cell layer then is extracted from each well with 0.5 ml of 1% Triton X-100. The cAMP levels then are determined using a radioimmunoassay kit (*e.g.*, Amersham, Arlington Heights, Illinois). Cells treated with OP-1 alone and with OP-1, the inhibitor, and a molecule capable of releasing morphogen inhibition have a significantly increased cAMP production in the presence of PTH compared to cells treated with OP-1 and the inhibitor.

### C. Induction of Osteocalcin Production

Osteocalcin is a bone-specific protein synthesized by osteoblasts which plays an integral role in determining the rate of bone mineralization *in vivo*. Circulating levels of osteocalcin in serum are used as a marker for osteoblast activity and bone formation *in vivo*. Induction of osteocalcin synthesis in osteoblast-enriched cultures can be used to demonstrate morphogenic efficacy *in vitro*.

Rat osteoblasts are prepared and cultured in a multi-well plate as described above. In this experiment the medium is supplemented with 10% FBS, and on day 2, cells are fed with fresh medium supplemented with fresh 10 mM  $\beta$ -glycerophosphate (Sigma, Inc.). Beginning on day 5 and twice weekly thereafter, cells are fed with a complete mineralization medium containing all of the above components plus fresh L(+)-ascorbate, at a final concentration of 50mg/ml medium.

OP-1 alone (*e.g.*, 0.1, 1.0, 10.0, 40.0 or 80.0 ng/ml), OP-1 and the inhibitor, or OP-1, the inhibitor, and various concentrations of a candidate compound are then added to the wells directly, *e.g.*, in 50% acetonitrile (or 50% ethanol) containing 0.1% trifluoroacetic acid (TFA), at no more than 5ml morphogen/ml medium. The cells then are re-fed and the conditioned medium sample diluted 1:1 in standard radioimmunoassay buffer containing standard protease inhibitors and stored at -20° C until assayed for osteocalcin. Osteocalcin synthesis is measured by standard radioimmunoassay using a commercially available osteocalcin-specific antibody and can be confirmed by Northern blot analysis to calculate the amount of osteocalcin mRNA produced in the presence and absence of OP-1 or morphogen analog. OP-1 induces a dose-dependent increase in osteocalcin production (5-fold increase using 25 ng of OP-1 protein/ml), and a 20-fold increase in osteocalcin mRNA. Cells treated with OP-1 alone and with OP-1, the inhibitor, and a

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molecule capable of releasing morphogen inhibition have a significant increase in osteocalcin production and osteocalcin mRNA compared to cells treated with OP-1 and the inhibitor.

Mineralization is determined on long term cultures (13 day) using a modified von Kossa staining technique on fixed cell layers: cells are fixed in fresh 4% paraformaldehyde at 23° C for 10 min, following rinsing with cold 0.9% NaCl. Fixed cells then are stained for endogenous alkaline phosphatase at pH 9.5 for 10 min, using a commercially available kit (Sigma, Inc.) Purple stained cells then are dehydrated with methanol and air dried. After 30 min incubation in 3% AgNO<sub>3</sub> in the dark, H<sub>2</sub>O-rinsed samples are exposed for 30 sec to 254 nm UV light to develop the black silver-stained phosphate nodules. Individual mineralized foci (at least 20 mm in size) are counted under a dissecting microscope and expressed as nodules/culture. OP-1 induces a 20-fold increase in initial mineralization rate. Cells treated with OP-1 alone and with OP-1, the inhibitor, and a molecule capable of releasing morphogen inhibition have a significant increase in initial mineralization rate compared to cells treated with OP-1 and the inhibitor.

#### D. *Inhibition of Epithelial Cell Growth by OP-1*

Morphogens are known to inhibit epithelial cells. Thus, the ability of OP-1 to inhibit cell proliferation, as measured by <sup>3</sup>H-thymidine uptake by an epithelial cell can be used in an assay to evaluate the ability of the candidate to release morphogen inhibition.

As an example, mink lung epithelial cell growth is inhibited by OP-1. (see, PCT US93/08885; WO 94/06420.) As described above, derivatives of these cells (*e.g.*, "R-4 mutants", clone 4-2, Laiho *et al.* (1990) *J. Biol. Chem.* 265: 18518-18524) can be transfected with DNA encoding OP-1-specific receptors and induced to express these receptors. The transfected cells, then can be assayed for a candidate analog's ability to block cell growth. As one example, when R-4 cells are transfected with ALK-1 under a Zn<sup>2+</sup>-inducible promoter, and induced to express the receptor following induction with ZnCl<sub>2</sub>, cell growth can be inhibited in the presence of OP-1 in a dose dependent manner. Cells treated with OP-1 alone and with OP-1, the inhibitor, and a molecule capable of releasing morphogen inhibition have a significant cell growth inhibition compared to cells treated with OP-1 and the inhibitor.

In a typical assay, cells are seeded in 24-well cell culture plates at a density of 104 cells per well in DMEM with 10% FBS, and incubated overnight. The medium is replaced with

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DMEM containing 0.2% FBS and 100  $\mu$ M ZnCl<sub>2</sub>, and the cells are incubated for 5 h, after which the medium is replaced with fresh DMEM containing 0.2% FBS, 100  $\mu$ M ZnCl<sub>2</sub> and various concentrations of OP-1 or an analog candidate. After 16 h of incubation, 0.25 Ci of <sup>3</sup>H-thymidine (Amersham) are added and the cells incubated for an additional 2 h. Thereafter, the cells are fixed  
5 in 10% trichloroacetic acid for more than 15 min on ice, and solubilized with 1 M NaOH. The cell extracts are neutralized with 1 M HCl and <sup>3</sup>H radioactivity determined in a liquid scintillation counter.

Candidates competent to release morphogen inhibition and restore the inhibition epithelial cell growth are contemplated to have particular utility in therapeutic applications where limitation  
10 of a proliferating cell population is desired. Such applications include chemotherapies and radiation therapies where limiting the growth of a normally proliferating population of cells can protect these cells from the cytotoxic effects of these cancer therapies. (see *e.g.*, WO 94/06420). In addition, psoriasis and other tissue disorders resulting from uncontrolled cell proliferation, including benign and malignant neoplasia, can be modulated by use of an OP-1 analog.

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What is claimed is:

- 1 1. A method for potentiating morphogen activity, comprising administering to a mammal a  
2 composition comprising a molecule capable of releasing morphogen inhibition.
- 1 2. A method for promoting neuronal cell growth, comprising administering to a mammal a  
2 composition comprising a molecule capable of releasing morphogen inhibition, thereby to  
3 potentiate growth-promoting effects of endogenous morphogens.
- 1 3. A method for treating a disorder characterized by neuronal cell loss, comprising  
2 administering to a mammal a composition comprising a molecule capable of releasing  
3 morphogen inhibition, thereby to potentiate growth-promoting effects of endogenous  
4 morphogens.
- 1 4. A method for treating a neurodegenerative disorder, comprising administering to a  
2 mammal a composition comprising a molecule capable of releasing morphogen inhibition.
- 1 5. The method of claim 1, wherein said morphogen activity is endogenous.
- 1 6. The method of claim 1, wherein said morphogen activity is the result of an exogenously  
2 provided morphogen.
- 1 7. The method of claim 4, wherein said composition further comprises a morphogen.
- 1 8. The method of claim 3 or 4, wherein said disorder is selected from the group consisting  
2 of: Alzheimer's disease, Parkinson's disease, Huntington's disease, senile dementia,  
3 alcohol-induced dementia, and stroke.
- 1 9. The method of claim 1, 2, 3 or 4, wherein said agent capable of releasing morphogen  
2 inhibition is selected from the group consisting of a cytokine antagonist, a retinoid  
3 antagonist, and a protein kinase A inhibitor.
- 1 10. The method of claim 9, wherein said cytokine antagonist is a neuropoetic cytokine  
2 antagonist.
- 1 11. The method of claim 10, wherein said neuropoetic cytokine antagonist is selected from the  
2 group consisting of an LIF antagonist and a CTNF antagonist.
- 1 12. The method of claim 11, wherein said LIF antagonist is monoclonal antibody to the gp130  
2 protein.

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- 1 13. The method of claim 9, wherein said retinoid antagonist is a retinoic acid receptor  
2 antagonist.
- 1 14. The method of claim 9, wherein said retinoid antagonist is a retinoid X receptor  
2 antagonist.
- 1 15. The method of claim 9, wherein said protein kinase A inhibitor is selected from the group  
2 consisting of (2-p-bromocynnamylaminoethyl) -5- isoquinolinesulfonamide, an enantiomer  
3 of dibutyryl cAMP, and an enantiomer of cAMP.
- 1 16. The method of claim 7, wherein said morphogen comprises an amino acid sequence  
2 selected from the group consisting of a sequence:
- 3 (a) having at least 70% homology with the C-terminal seven-cysteine skeleton of  
4 human OP-1, residues 330-431 of SEQ ID NO: 2;
- 5 (b) having greater than 60% amino acid sequence identity with said C-terminal  
6 seven-cysteine skeleton of human OP-1;
- 7 (c) defined by Generic Sequence 7, SEQ ID NO: 4;
- 8 (d) defined by Generic Sequence 8, SEQ ID NO: 5;
- 9 (e) defined by Generic Sequence 9, SEQ ID NO: 6;
- 10 (f) defined by Generic Sequence 10, SEQ ID NO: 7; and
- 11 (g) defined by OPX, SEQ ID NO: 3.
- 1 17. The method of claim 7, wherein said morphogen is selected from the group consisting of  
2 human OP-1, mouse OP-1, human OP-2, mouse OP-2, 60A, GDF-1, BMP2A, BMP2B,  
3 DPP, Vgl, Vgr-1, BMP3, BMP5, and BMP6.
- 1 18. The method of claim 7, wherein said morphogen is OP-1.
- 1 19. A method for potentiating morphogen activity comprising the step of  
2 administering to a mammal a composition comprising a molecule that binds an  
3 endogenous ligand for a receptor selected from the group consisting of a cytokine  
4 receptor and a retinoid receptor.
- 1 20. The method of claim 19, wherein said cytokine receptor is a neuropoetic cytokine  
2 receptor.

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- 1 21. The method of claim 20, wherein said neuropoetic cytokine receptor is selected from the  
2 group consisting of an LIF receptor and a CTNF receptor.
- 1 22. The method of claim 19, wherein said retinoid receptor is a retinoic acid receptor.
- 1 23. The method of claim 19, wherein said retinoid receptor is a retinoid X receptor.
- 1 24. A method for potentiating morphogen activity comprising the step of  
2 administering to a mammal a composition comprising a cAMP-dependent  
3 messenger pathway inhibitor.
- 1 25. The method of claim 24, wherein said cAMP-dependent messenger pathway  
2 inhibitor comprises a protein kinase A inhibitor.
- 1 26. The method of claim 25, wherein said protein kinase A inhibitor is selected from the group  
2 consisting of (2-p-bromocinnamylaminoethyl) -5- isoquinolinesulfonamide, an enantiomer  
3 of dibutyryl cAMP, and an enantiomer of cAMP.
- 1 27. A screening method for identifying a molecule capable of potentiating morphogen activity,  
2 comprising the steps of
- 3 (1) providing a test cell comprising a morphogen inhibitory element, said cell, when  
4 contacted with OP-1, not undergoing tissue morphogenesis;
- 5 (2) exposing said test cell to OP-1 and a candidate molecule; and
- 6 (3) identifying a molecule capable of potentiating morphogen activity as a candidate  
7 that releases morphogen inhibition permitting said cell to undergo OP-1-induced  
8 tissue morphogenesis.
- 1 28. The screening method of claim 27, wherein said test cell is selected from the group  
2 consisting of sympathetic nerves, hippocampus, cerebral cortex, striatum, kidney, liver,  
3 adrenals, urinary bladder, and testes.
- 1 29. A molecule identified by the method of claim 27.
- 1 30. The molecule of claim 29, wherein said molecule is a protein.
31. The molecule of claim 29, wherein said molecule is an inorganic molecule.
32. The molecule of claim 29, wherein said molecule is an organic molecule.

## % Sequence Similarity to Human OP-1 Seven-Cysteine Domain

Sequence	% Similarity	% Non Conservative
hOP-1	100	0
mOP-1	100	0
hOP-2	97	3
mOP-2	97	3
BMP-5	97	3
BMP-6	96	4
Vgr-1(PT)	94	6
OP-3	91	9
60A	90	10
BMP-4	90	10
BMP-2	89	11
dpp	87	13
UNIVIN	87	13
dpp(PT)	86	14
Vg-1	86	14
CDMP-1	85	15
CDMP-3	83	17
GDF-3	83	17
CDMP-2	82	18
DORSALIN	79	21
GDF-1(PT)	78	22
GDF-10	78	22
BMP-3b	78	22
BMP-10	78	23
BMP-3	78	23
SCREW	77	23
ADMP	77	24
TGF- $\beta$ 2	73	27
GDF-1	73	28
BMP-9	73	28
NODAL	71	29
Inhibin $\beta$ A	71	29
BMP-15	71	29
TGF- $\beta$ 3	69	31
Inhibin $\beta$ B	69	31
Inhibin $\beta$ C	69	31
TGF- $\beta$ 5	67	33
TGF- $\beta$ 1	67	33
GDF-12	67	33
GDF-11	66	34
TGF- $\beta$ 4	66	34
GDF-9	66	34
GDF-8	64	36
BMP-11	60	40
GDNF	49	51

FIG. 1

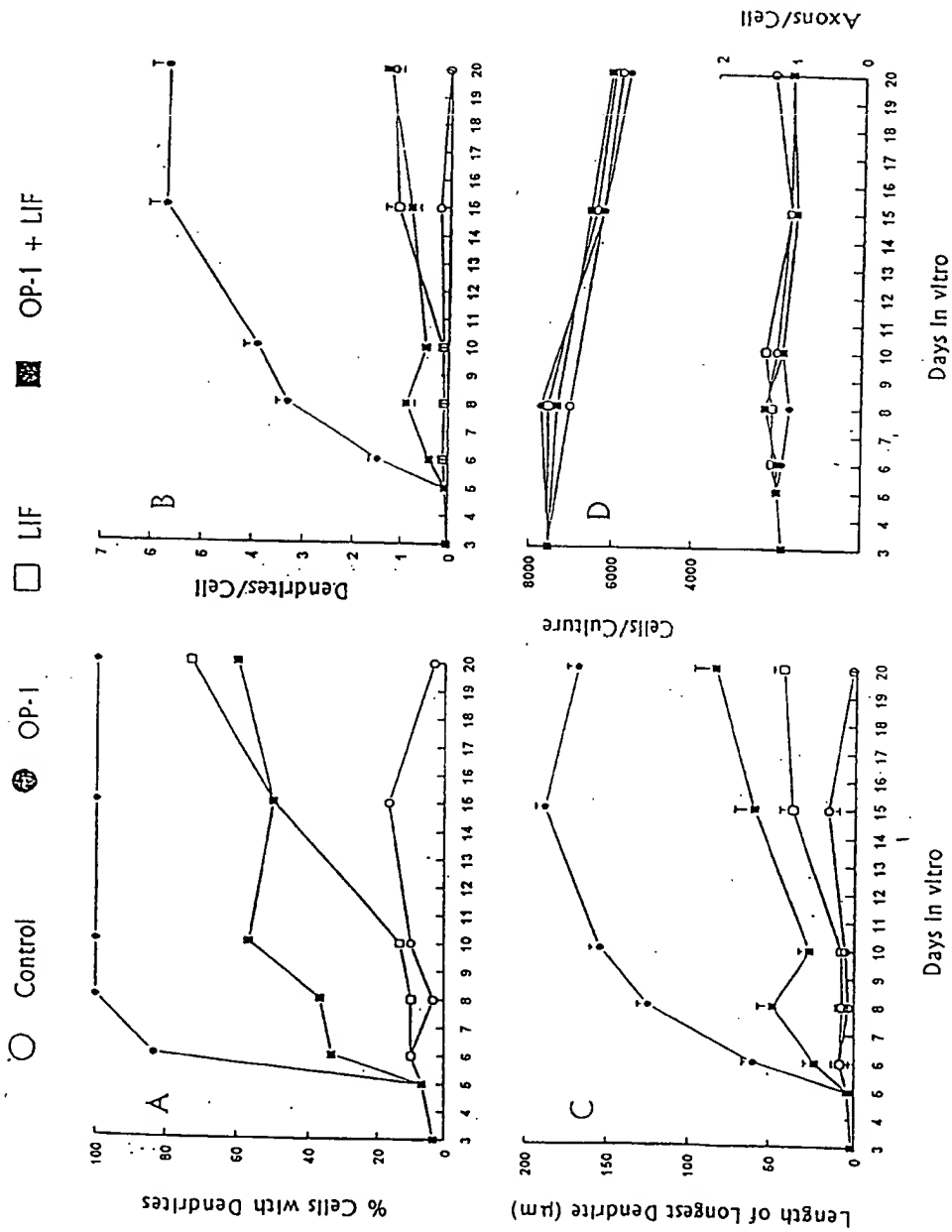


Fig. 2



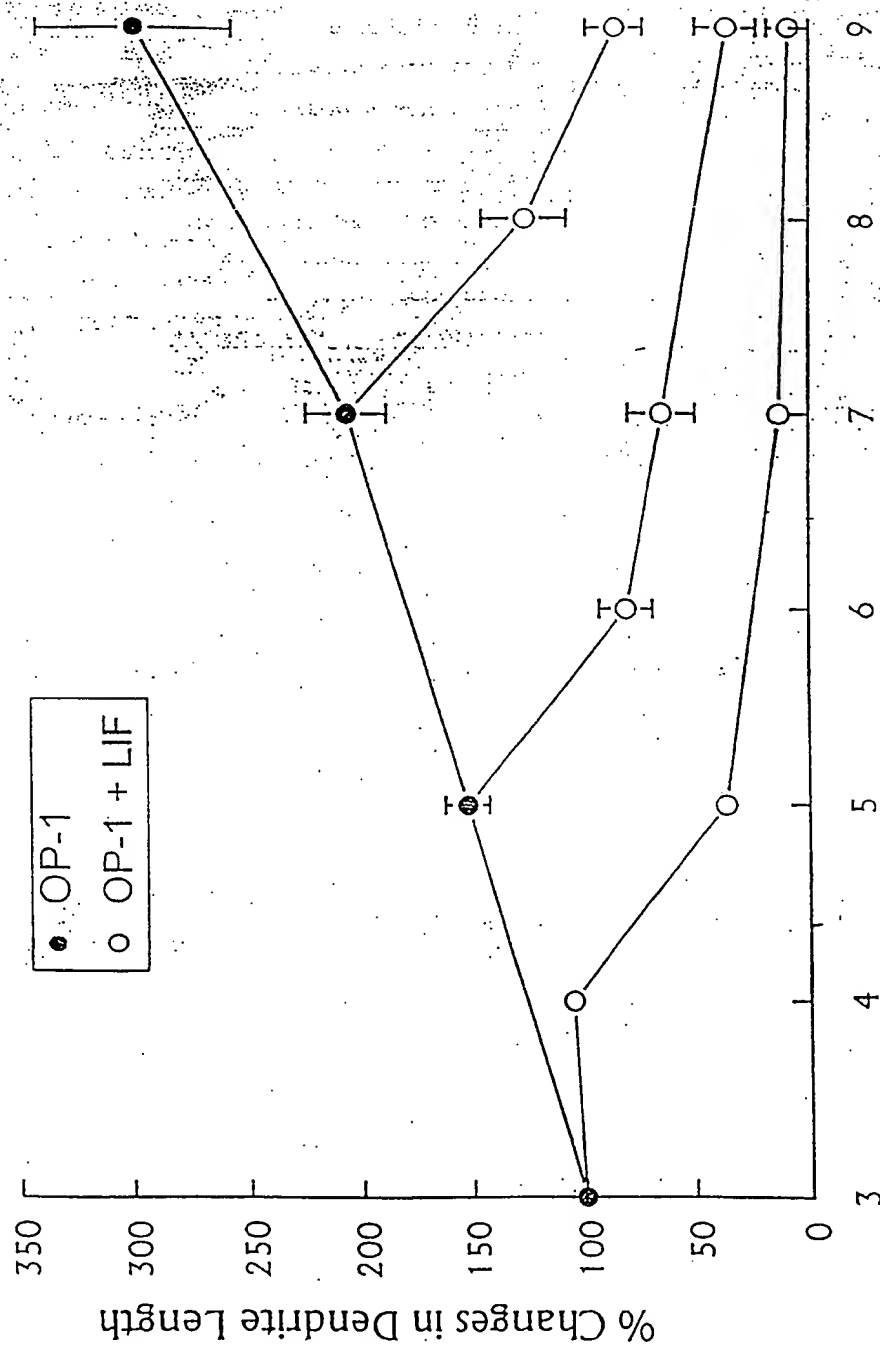
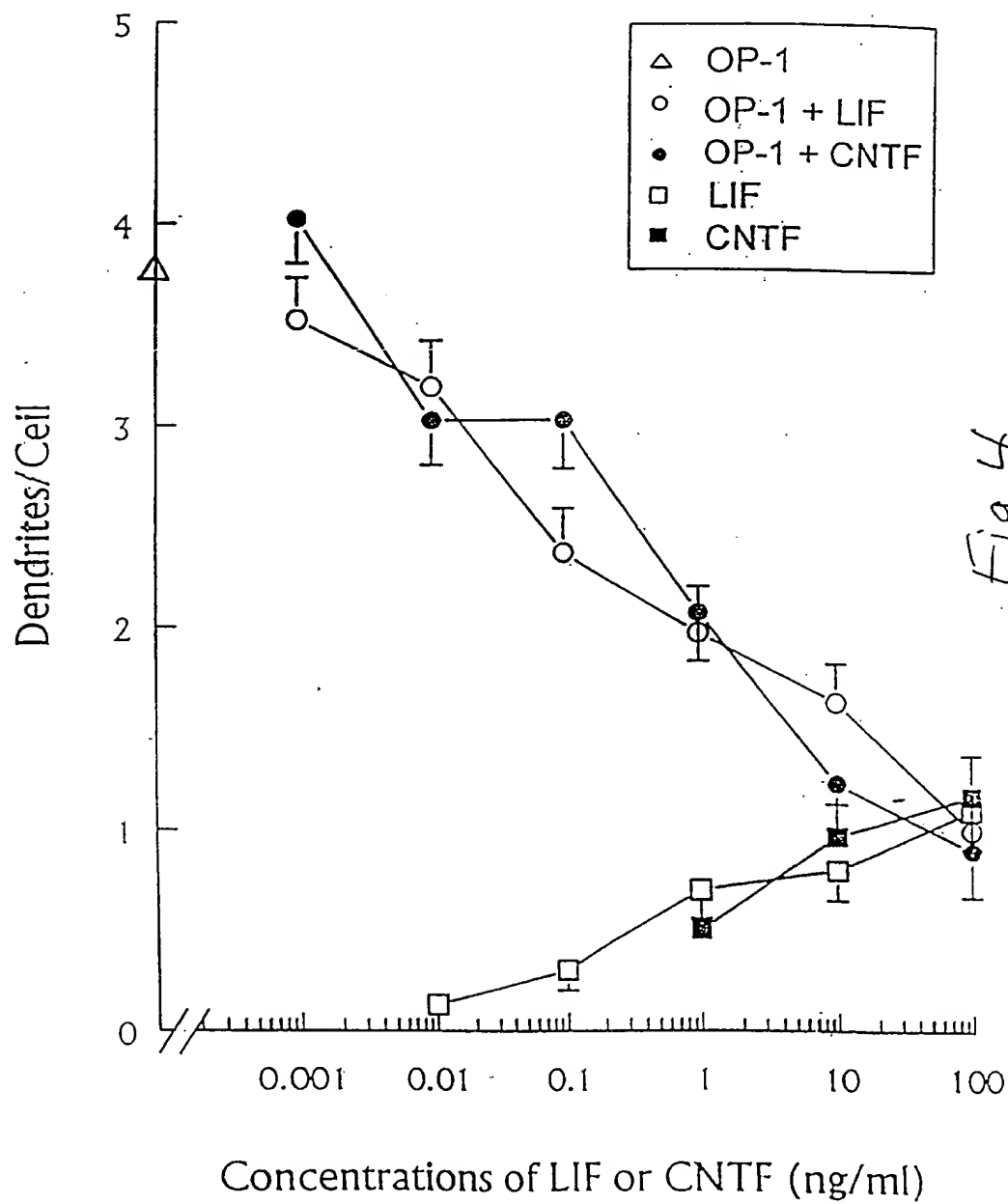


Fig. 3

Days in OP-1



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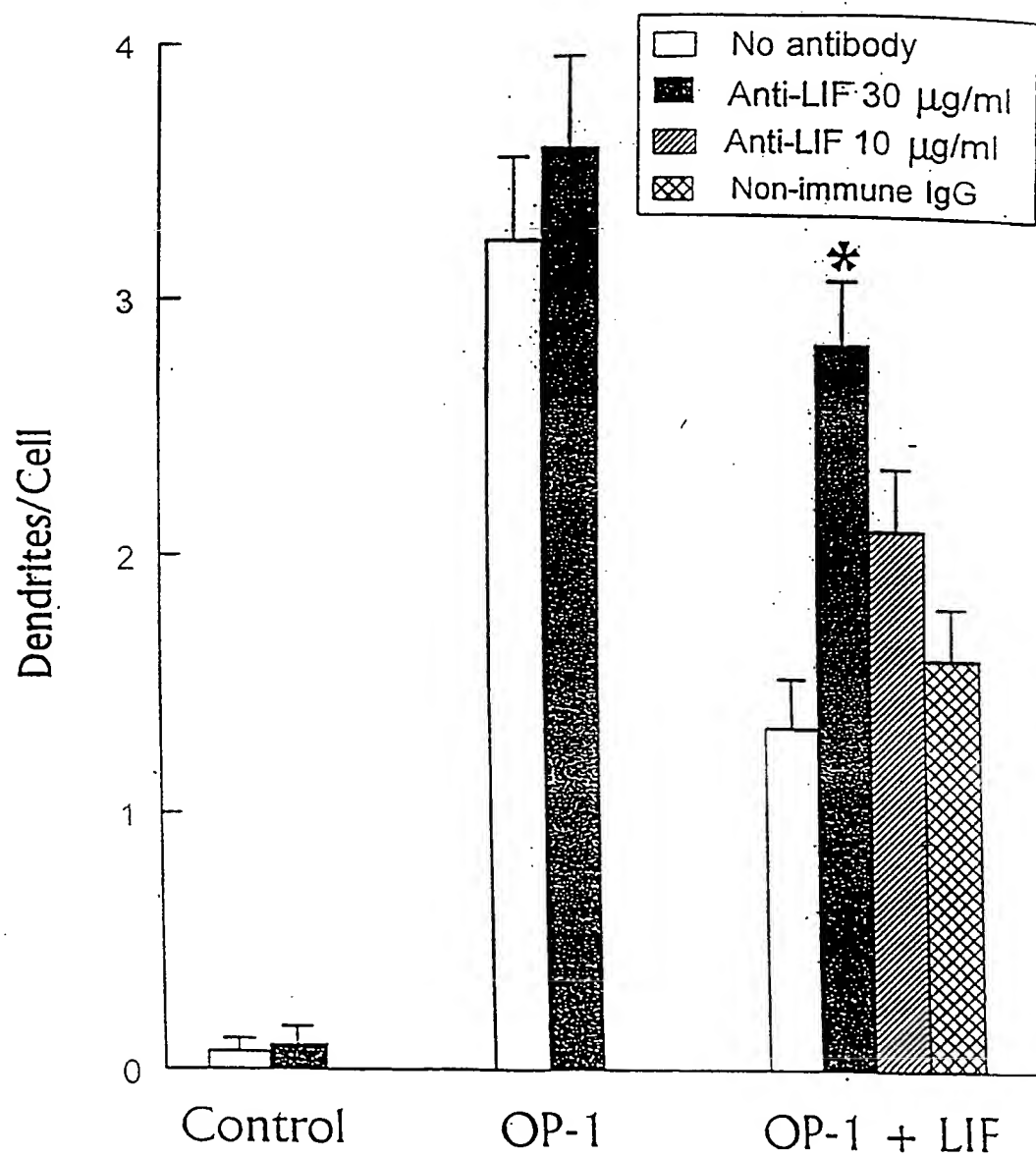


Fig. 5

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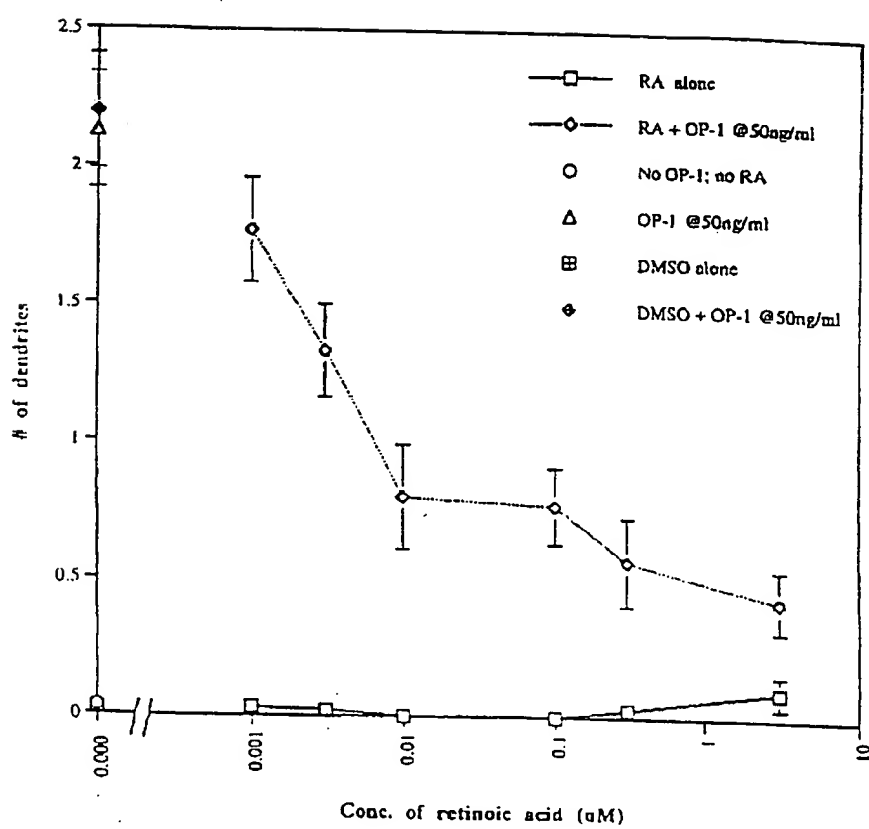


Fig. 6

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## Dose dependent inhibition of dendritic growth by retinoic acid

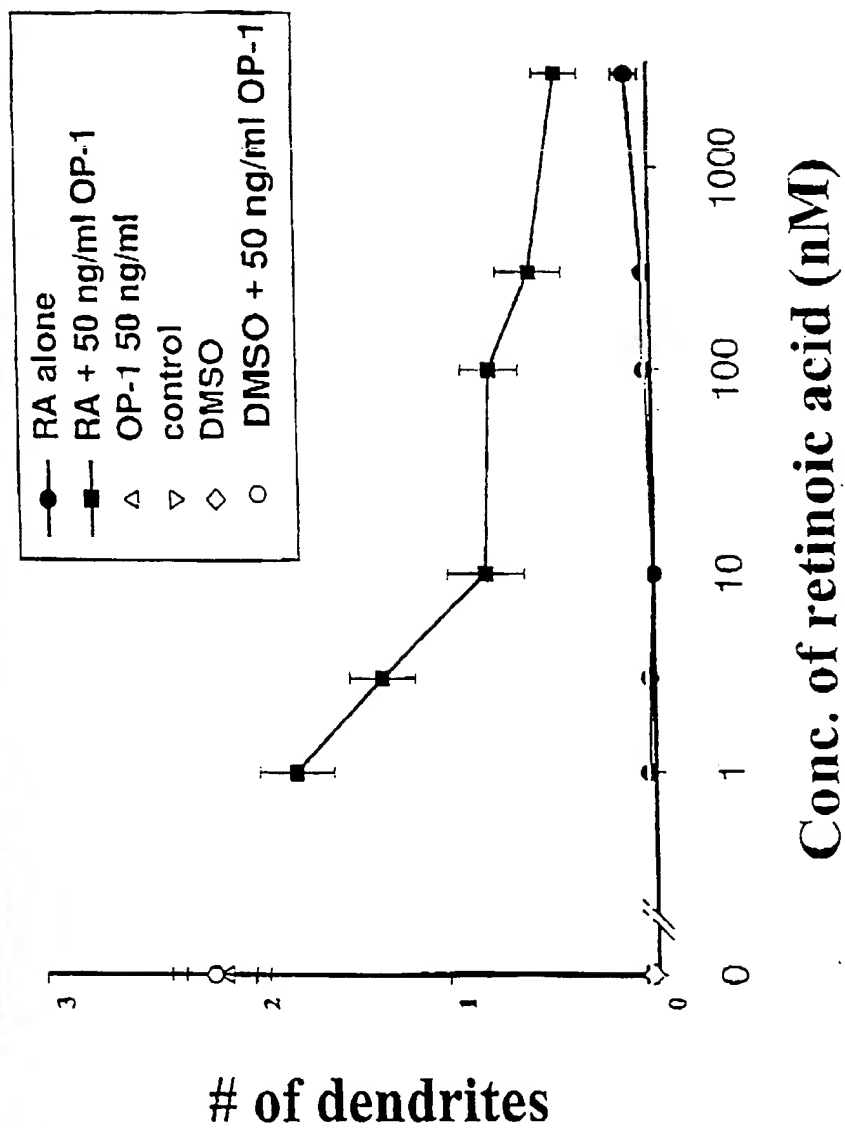


FIG. 8A

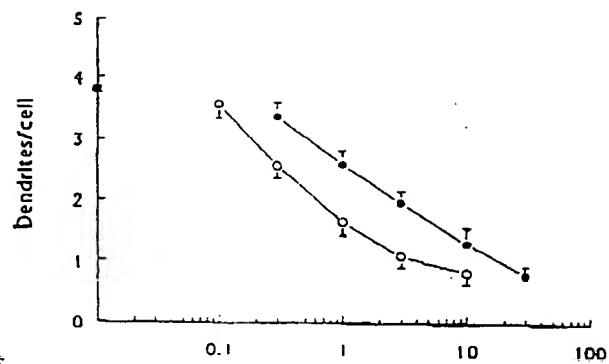


FIG. 8B

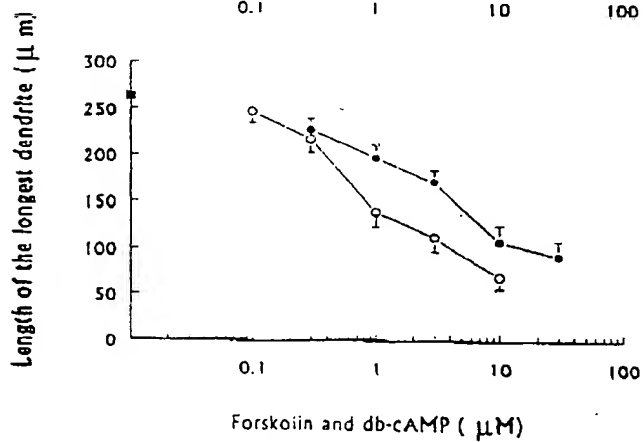
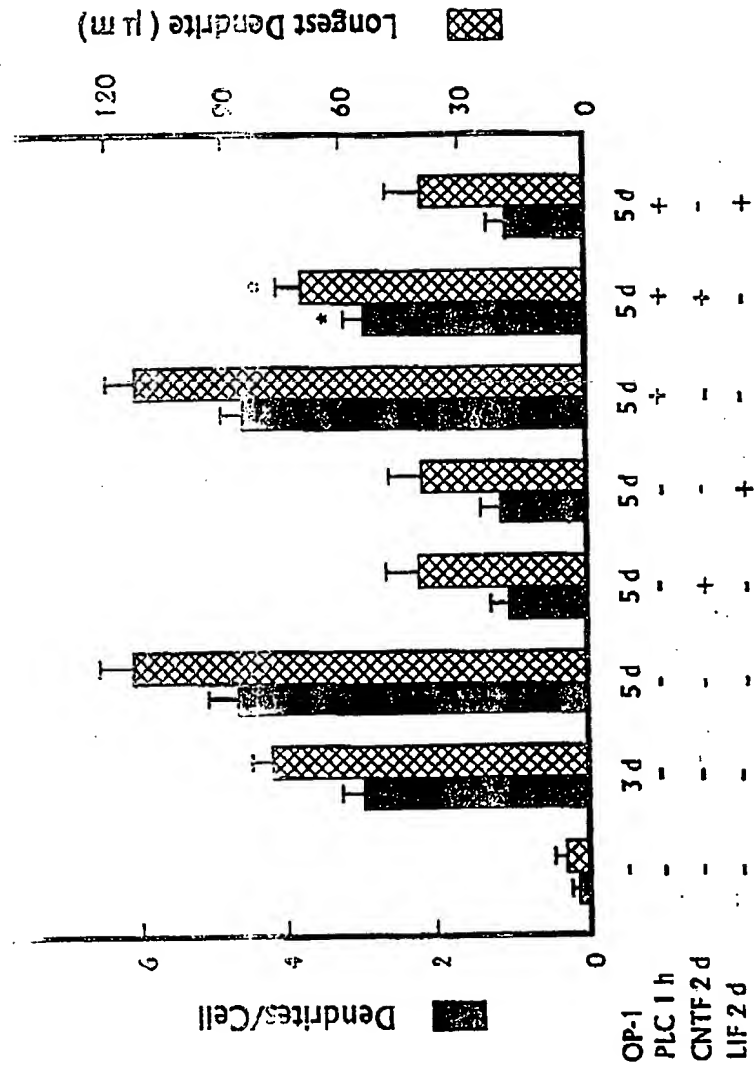


FIG. 9



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: CHARETTE, MARC F.  
RUEGER, DAVID C.  
HIGGINS, DENNIS
- (ii) TITLE OF INVENTION: ENHANCEMENT OF MORPHOGEN ACTIVITY
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: PATENT ADMINISTRATOR, TESTA, HURWITZ &  
THIBEAULT, LLP  
(B) STREET: 125 HIGH STREET  
(C) CITY: BOSTON  
(D) STATE: MA  
(E) COUNTRY: USA  
(F) ZIP: 02110
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: MEYERS, THOMAS C.  
(B) REGISTRATION NUMBER: 36,989  
(C) REFERENCE/DOCKET NUMBER: CRP-158PR
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (617) 248-7000  
(B) TELEFAX: (617) 248-7100

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1822 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:  
(A) ORGANISM: HOMO SAPIENS  
(F) TISSUE TYPE: HIPPOCAMPUS
- (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 49..1341  
(C) IDENTIFICATION METHOD: experimental  
(D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"  
/product= "OP1"  
/evidence= EXPERIMENTAL  
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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGC TCA CTG CGA GCT GCG GCG CCG CAC AGC TTC GTG GCG CTC TGG GCA      105
Arg Ser Leu Arg Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala
   5                10                15
CCC CTG TTC CTG CTG CGC TCC GCC CTG GCC GAC TTC AGC CTG GAC AAC      153
Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn
  20                25                30                35
GAG GTG CAC TCG AGC TTC ATC CAC CGG CGC CTC CGC AGC CAG GAG CGG      201

```



Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg	
40 45 50	
CGG GAG ATG CAG CGC GAG ATC CTC TCC ATT TTG GGC TTG CCC CAC CGC	249
Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg	
55 60 65	
CCG CGC CCG CAC CTC CAG GGC AAG CAC AAC TCG GCA CCC ATG TTC ATG	297
Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met	
70 75 80	
CTG GAC CTG TAC AAC GCC ATG GCG GTG GAG GAG GGC GGC GGG CCC GGC	345
Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly Gly Pro Gly	
85 90 95	
GGC CAG GGC TTC TCC TAC CCC TAC AAG GCC GTC TTC AGT ACC CAG GGC	393
Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr Gln Gly	
100 105 110 115	
CCC CCT CTG GCC AGC CTG CAA GAT AGC CAT TTC CTC ACC GAC GCC GAC	441
Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala Asp	
120 125 130	
ATG GTC ATG AGC TTC GTC AAC CTC GTG GAA CAT GAC AAG GAA TTC TTC	489
Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu Phe Phe	
135 140 145	
CAC CCA CGC TAC CAC CAT CGA GAG TTC CGG TTT GAT CTT TCC AAG ATC	537
His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile	
150 155 160	
CCA GAA GGG GAA GCT GTC ACG GCA GCC GAA TTC CGG ATC TAC AAG GAC	585
Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Asp	
165 170 175	
TAC ATC CGG GAA CGC TTC GAC AAT GAG ACG TTC CGG ATC AGC GTT TAT	633
Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile Ser Val Tyr	
180 185 190 195	
CAG GTG CTC CAG GAG CAC TTG GGC AGG GAA TCG GAT CTC TTC CTG CTC	681
Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu Phe Leu Leu	
200 205 210	
GAC AGC CGT ACC CTC TGG GCC TCG GAG GAG GGC TGG CTG GTG TTT GAC	729
Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu Val Phe Asp	
215 220 225	
ATC ACA GCC ACC AGC AAC CAC TGG GTG GTC AAT CCG CGG CAC AAC CTG	777
Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg His Asn Leu	
230 235 240	
GGC CTG CAG CTC TCG GTG GAG ACG CTG GAT GGG CAG AGC ATC AAC CCC	825
Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile Asn Pro	
245 250 255	
AAG TTG GCG GGC CTG ATT GGG CGG CAC GGG CCC CAG AAC AAG CAG CCC	873
Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys Gln Pro	
260 265 270 275	
TTC ATG GTG GCT TTC TTC AAG GCC ACG GAG GTC CAC TTC CGC AGC ATC	921
Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe Arg Ser Ile	
280 285 290	
CGG TCC ACG GGG AGC AAA CAG CGC AGC CAG AAC CGC TCC AAG ACG CCC	969
Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro	
295 300 305	
AAG AAC CAG GAA GCC CTG CGG ATG GCC AAC GTG GCA GAG AAC AGC AGC	1017
Lys Asn Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser	
310 315 320	
AGC GAC CAG AGG CAG GCC TGT AAG AAG CAC GAG CTG TAT GTC AGC TTC	1065
Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe	
325 330 335	
CGA GAC CTG GGC TGG CAG GAC TGG ATC ATC GCG CCT GAA GGC TAC GCC	1113
Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Pro Glu Gly Tyr Ala	
340 345 350 355	
GCC TAC TAC TGT GAG GGG GAG TGT GCC TTC CCT CTG AAC TCC TAC ATG	1161
Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met	

360								365				370				
AAC Asn	GCC Ala	ACC Thr	AAC Asn 375	CAC His	GCC Ala	ATC Ile	GTG Val	CAG Gln 380	ACG Thr	CTG Leu	GTC Val	CAC His	TTC Phe 385	ATC Ile	AAC Asn	1209
CCG Pro	GAA Glu	ACG Thr 390	GTG Val	CCC Pro	AAG Lys	CCC Pro	TGC Cys 395	TGT Cys	GCG Ala	CCC Pro	ACG Thr	CAG Gln 400	CTC Leu	AAT Asn	GCC Ala	1257
ATC Ile	TCC Ser 405	GTC Val	CTC Leu	TAC Tyr	TTC Phe	GAT Asp 410	GAC Asp	AGC Ser	TCC Ser	AAC Asn	GTC Val 415	ATC Ile	CTG Leu	AAG Lys	AAA Lys	1305
TAC Tyr 420	AGA Arg	AAC Asn	ATG Met	GTG Val 425	GTC Val	CGG Arg	GCC Ala	TGT Cys	GGC Gly	TGC Cys 430	CAC His	TAGCTCCTCC			1351	
GAGAATT CAG ACCCTTTGGG GCCAAGTTTT TCTGGATCCT CCATTGCTCG CCTTGGCCAG 1411																
GAACCAGCAG ACCAACTGCC TTTTGTGAGA CTTTCCCCTC CCTATCCCCA ACTTTAAAGG 1471																
TGTGAGAGTA TTAGGAAACA TGAGCAGCAT ATGGCTTTTG ATCAGTTTTT CAGTGGCAGC 1531																
ATCCAATGAA CAAGATCCTA CAAGCTGTGC AGGCAAAACC TAGCAGGAAA AAAAAACAAC 1591																
GCATAAAGAA AAATGGCCGG GCCAGGTCAT TGGCTGGGAA GTCTCAGCCA TGCACGGACT 1651																
CGTTTCCAGA GGTAATTATG AGCGCCTACC AGCCAGGCCA CCCAGCCGTG GGAGGAAGGG 1711																
GGCGTGGCAA GGGGTGGGCA CATTGGTGTG TGTGCGAAAG GAAAATTGAC CCGGAAGTTC 1771																
CTGTAATAAA TGTCACAATA AAACGAATGA ATGAAAAAAA AAAAAAAAAA A 1822																

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 431 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met <sub>1</sub>	His	Val	Arg	Ser <sub>5</sub>	Leu	Arg	Ala	Ala	Ala <sub>10</sub>	Pro	His	Ser	Phe	Val <sub>15</sub>	Ala
Leu	Trp	Ala	Pro <sub>20</sub>	Leu	Phe	Leu	Leu	Arg <sub>25</sub>	Ser	Ala	Leu	Ala	Asp <sub>30</sub>	Phe	Ser
Leu	Asp	Asn <sub>35</sub>	Glu	Val	His	Ser	Ser <sub>40</sub>	Phe	Ile	His	Arg	Arg <sub>45</sub>	Leu	Arg	Ser
Gln	Glu <sub>50</sub>	Arg	Arg	Glu	Met	Gln <sub>55</sub>	Arg	Glu	Ile	Leu	Ser <sub>60</sub>	Ile	Leu	Gly	Leu
Pro <sub>65</sub>	His	Arg	Pro	Arg	Pro <sub>70</sub>	His	Leu	Gln	Gly <sub>75</sub>	His	Asn	Ser	Ala	Pro <sub>80</sub>	
Met	Phe	Met	Leu	Asp <sub>85</sub>	Leu	Tyr	Asn	Ala	Met <sub>90</sub>	Ala	Val	Glu	Glu	Gly <sub>95</sub>	Gly
Gly	Pro	Gly	Gly <sub>100</sub>	Gln	Gly	Phe	Ser	Tyr <sub>105</sub>	Pro	Tyr	Lys	Ala	Val <sub>110</sub>	Phe	Ser
Thr	Gln	Gly <sub>115</sub>	Pro	Pro	Leu	Ala	Ser <sub>120</sub>	Leu	Gln	Asp	Ser	His <sub>125</sub>	Phe	Leu	Thr
Asp	Ala <sub>130</sub>	Asp	Met	Val	Met	Ser <sub>135</sub>	Phe	Val	Asn	Leu	Val <sub>140</sub>	Glu	His	Asp	Lys
Glu <sub>145</sub>	Phe	Phe	His	Pro	Arg <sub>150</sub>	Tyr	His	His	Arg	Glu <sub>155</sub>	Phe	Arg	Phe	Asp	Leu <sub>160</sub>
Ser	Lys	Ile	Pro	Glu <sub>165</sub>	Gly	Glu	Ala	Val	Thr <sub>170</sub>	Ala	Ala	Glu	Phe	Arg <sub>175</sub>	Ile
Tyr	Lys	Asp	Tyr <sub>180</sub>	Ile	Arg	Glu	Arg	Phe <sub>185</sub>	Asp	Asn	Glu	Thr	Phe <sub>190</sub>	Arg	Ile

Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu  
 195 200 205  
 Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu  
 210 215 220  
 Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg  
 225 230 235 240  
 His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser  
 245 250 255  
 Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn  
 260 265 270  
 Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe  
 275 280 285  
 Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser  
 290 295 300  
 Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu  
 305 310 315 320  
 Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr  
 325 330 335  
 Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu  
 340 345 350  
 Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn  
 355 360 365  
 Ser Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His  
 370 375 380  
 Phe Ile Asn Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln  
 385 390 395 400  
 Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile  
 405 410 415  
 Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His  
 420 425 430

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 102 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (ix) FEATURE:  
 (A) NAME/KEY: Protein  
 (B) LOCATION: 1..102  
 (D) OTHER INFORMATION: /label= OPX

/note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Xaa Xaa His Glu Leu Tyr Val Xaa Phe Xaa Asp Leu Gly Trp Xaa  
 1 5 10 15  
 Asp Trp Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly  
 20 25 30  
 Glu Cys Xaa Phe Pro Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala  
 35 40 45  
 Ile Xaa Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa Xaa Val Pro Lys  
 50 55 60  
 Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa

5

```

65           70           75           80
Asp Xaa Ser Xaa Asn Val Xaa Leu Xaa Lys Xaa Arg Asn Met Val Val
      85           90           95
Xaa Ala Cys Gly Cys His
      100

```

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 97 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein  
 (B) LOCATION: 1..97

(D) OTHER INFORMATION: /label= Generic-Seq-7

/note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Leu Xaa Xaa Xaa Phe Xaa Xaa Xaa Gly Trp Xaa Xaa Xaa Xaa Xaa
1           5           10           15
Pro Xaa Xaa Xaa Xaa Ala Xaa Tyr Cys Xaa Gly Xaa Cys Xaa Xaa Pro
      20           25           30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asn His Ala Xaa Xaa Xaa Xaa Xaa
      35           40           45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Cys Xaa Pro
      50           55           60
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa
      65           70           75           80
Val Xaa Leu Xaa Xaa Xaa Xaa Xaa Met Xaa Val Xaa Xaa Cys Xaa Cys
      85           90           95
Xaa

```

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 102 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein  
 (B) LOCATION: 1..102

(D) OTHER INFORMATION: /label= Generic-Seq-8

/note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Cys Xaa Xaa Xaa Xaa Leu Xaa Xaa Xaa Phe Xaa Xaa Xaa Gly Trp Xaa
1           5           10           15
Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa Xaa Ala Xaa Tyr Cys Xaa Gly
      20           25           30
Xaa Cys Xaa Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asn His Ala
      35           40           45

```

```

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 50                      55                      60
Xaa Cys Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65                      70                      75                      80
Xaa Xaa Xaa Xaa Xaa Val Xaa Leu Xaa Xaa Xaa Xaa Xaa Met Xaa Val
                      85                      90                      95
Xaa Xaa Cys Xaa Cys Xaa
                      100

```

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 97 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein  
 (B) LOCATION: 1..97  
 (D) OTHER INFORMATION: /label= Generic-Seq-9

/note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1                      5                      10                      15
Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly Xaa Cys Xaa Xaa Xaa
20                      25                      30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35                      40                      45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Pro
50                      55                      60
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65                      70                      75                      80
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys
85                      90                      95
Xaa

```

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 102 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: Protein  
 (B) LOCATION: 1..102  
 (D) OTHER INFORMATION: /label= Generic-Seq-10

/note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1                      5                      10                      15

```

7

Xaa Xaa Xaa Xaa Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Gly  
                   20                  25                  30  
 Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
           35                  40                  45  
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
           50                  55                  60  
 Xaa Xaa Cys Xaa Pro Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
   65                  70                  75                  80  
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
           85                  90                  95  
 Xaa Xaa Cys Xaa Cys Xaa  
                   100

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (ix) FEATURE:
  - (A) NAME/KEY: Protein
  - (B) LOCATION: 1..5
  - (D) OTHER INFORMATION: /note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Cys Xaa Xaa Xaa Xaa  
   1                  5

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (ix) FEATURE:
  - (A) NAME/KEY: Protein
  - (B) LOCATION: 1..5
  - (D) OTHER INFORMATION: /note= "wherein each Xaa is independently selected from a group of one or more specified amino acids as defined in the specification"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Cys Xaa Xaa Xaa Xaa  
   1                  5